STUDIES ON SULPHOTRANSFERASES

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June 1967

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. A.B. Roy for supervising the work with an unfailing interest.

Grateful thanks are also due to Professor A.G. Ogston for providing facilities to carry out the work and for agreeing to supervise during Dr. A.B. Roy's absence from Canberra from November 1965 to July, 1966.

Acknowledgement is also due to Mr. R.A. Goodman for assisting in the procurement of chemicals and apparatus and to Mrs. E. Allen for carrying out electrophoresis on starch gel as mentioned in Chapter 4.

I am thankful to Mrs. J. Whitelock for typing the thesis.

Financial support in the form of a research scholarship by the Australian National University is gratefully acknowledged.

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ABBREVIATIONS

The abbreviations used in this thesis are:-

ATP	=	adenosine 5'-triphosphate
APS	=	adenosine 5'-phosphosulphate
PAPS	=	3'-phosphoadenosine-5'-phosphosulphate or 3'-phosphoadenylyl sulphate
NP	=	p-nitrophenol
NPS	=	p-nitrophenyl sulphate
PAP	=	adenosine-3',5'-diphosphate
ADP	=	adenosine-5'-diphosphate

CHAPTER I

INTRODUCTION

Discovery of sulphate conjugates in animal body

Two groups of sulphate conjugates of biological interest are recognized. In the first group the sulphate is bound to a phenolic or alcoholic hydroxyl group forming ester sulphates or O-sulphates and in the second group the sulphate is bound to an amino group forming a sulphamate or N-sulphate.

The formation of sulphate conjugates in the animal body was first detected by Baumann (1876a, b, and 1878-79) who isolated the potassium salt of phenyl sulphate from human urine and also showed that the administration of various other phenols was followed by the urinary excretion of the corresponding aryl sulphates. This synthesis of aryl sulphates was observed in all the higher animals tested (Williams, 1959). The formation of these aryl sulphates provided an important pathway by which phenols were removed from the body, for in addition to being less toxic than their parent phenols, these sulphates were readily excreted with the urine. This therefore opened up a new field of study on the

general detoxication mechanisms in the animal body. It was found by Embden & Glässner (1902) and Herter & Wakeman (1899) that phenol conjugation was apparently a general function of many tissues with liver and intestine the predominant sites for this activity. Of the aryl sulphates, the classical phenyl sulphate combined with indoxyl sulphate and p-cresyl sulphate quantitatively forms the largest group of ester sulphates in the urine. The occurrence of these compounds in the urine has long been attributed to the bacterial degradation of aromatic amino acids in the intestines followed by subsequent sulphate conjugation (Williams, 1959). One interesting aryl sulphate, which according to Tallan, Bella, Stein & Moore (1955) occurs in the human urine, is tyrosine O-sulphate. It has subsequently been found in the urine of many mammalian species (John, Rose, Wusteman & Dodgson, 1966) and is a constituent of mammalian fibrinogens (Doolittle & Blombäck, 1964) and of Gastrin II from the pig (Gregory, Hardy, Jones, Kenner & Sheppard, 1964).

As the investigation of the aryl sulphates *in vivo* proceeded, steroid sulphates in the mammalian urine were also being recognized. Three main types

of steroid sulphates formed this important group of urinary ester sulphates in which the sulphate group is bound to the phenolic hydroxyl group as in oestrone (Schachter and Marrian, 1938), to a secondary hydroxyl group in the 3- position as in androstenolone (Venning, Hoffman & Browne, 1942; Baulieu & Michaud, 1961) or to a secondary hydroxyl group in the 17- position as in testosterone (Dessypris, Drosdowski, McNnen & Dorfman, 1966) and to a primary hydroxyl group in the 21- position as in desoxycorticosterone. Since the secondary alcoholic group in the steroids could occur in the α- or β- configurations the number of possible types of steroid sulphates increases further. Moreover the possibility of the formation of disulphates produced from dihydroxy steroids remains and Wengle & Bostrŏm (1963) were able to isolate the disulphate of androst-5-en-3β,17β-diol. These authors were also able to deduce certain structural features of the steroids which influenced the sulphurylation rates. For example, they showed that a double bond between carbon atoms 4 and 5 inhibited sulphurylation at the 3β-position, that 17β-sulphurylation was inhibited by a 17α-substitution by a methyl or ethyl

group and that a 17α-hydroxy group inhibited sulphurylation at the 21- position. These results correlate well with in vivo studies of steroid metabolism.

The existence of a third group of ester sulphates, the alkyl sulphates (Boström & Vestermark, 1960) was soon recognized and it was thought that this pathway of detoxication is quantitatively unimportant in the case of short chain aliphatic alcohols but is significant in the case of both the long chain aliphatic and the alicyclic alcohols (Vestermark & Boström, 1960).

The other group of sulphate esters constitute the very important series of compounds namely the sulphated carbohydrates. The classical chondroitin sulphates, keratosulphates and heparitins belong to this series. They are found protein-bound in the connective tissues and mucous membranes. In the chondroitin sulphates and the heparitins the sulphate group is bound to the various hydroxyl group of the hexosamine residues of the molecule (Hoffman, Linker & Meyer, 1958), whereas in the keratosulphates, which contain glucuronic acid and galactose, the sulphate group

is bound to the hydroxyl group of the galactose residues. The urinary excretion of the sulphomucopolysaccharides has currently become of importance as the increase in their excretion has been implicated in diseases like rheumatoid arthritis and psoriasis (diFerrante, 1957; Brunish & Sørensen, 1965).

The sulphamates or the N-sulphates form only a small group of sulphate conjugates of which heparin is the most well known physiological member (Jorpes, Boström & Mutt, 1950). Boyland, Manson & Orr (1957) then showed that certain arylamines administered to experimental animals are partially excreted as sulphamates. This was followed by the demonstration of the synthesis in vitro of 2-naphthyl sulphamate by rat liver preparations (Roy, 1958) and of phenyl sulphamate by enzymes from sheep intestinal mucosa (Kent & Pasternak, 1958). The detoxication of 2-naphthylamine by sulphate conjugation is important since 2-naphthylamine is known to cause bladder cancer in workers engaged in its manufacture.

Source of sulphur for conjugation

With the large number of metabolically formed sulphate conjugates as discussed above, it is now appropriate to consider the source of sulphur available for conjugation. Although it was believed that sulphate ions directly reacted to form sulphate conjugates *in vivo*, the observation of Rhode (1923) that the subcutaneous injection of either sodium sulphite or of cystine into rabbits receiving phenol orally caused an increase in the urinary phenyl sulphate but that sodium sulphate was ineffective, started an initial controversy. Hele (1924, 1931) subsequently showed that orally administered sodium sulphate is utilized in the formation of sulphate esters of phenol and indole and he suggested that the sulphate ions reacting can be both endogenous or exogenous, the former being produced by the catabolism of proteins and the latter being the administered sulphate ions themselves. Support for the view that exogenous sulphate can be used for sulphate conjugation came from the experiments of Bernheim & Bernheim (1943) who showed that guinea pig liver slices conjugated phenol only in the presence of sulphate ions and that the conjugation was abolished in the absence of free

sulphate. The slices could not utilize cystine or methionine as sources of sulphur.

Later work by Laidlaw & Young (1948, 1953) showed that when ^{35}S labelled sodium sulphate was injected into rats with a simultaneous administration of 2-naphthol, a high proportion of the ^{35}S was introduced into the resultant 2-naphthyl sulphuric acid in the urine.

While the above work showed that administered inorganic sulphate can participate in sulphate conjugation, they did not show that dietary sulphate, which is only poorly absorbed from the intestines, is the normal source of sulphur for these reactions.

Binkley (1949) showed that the addition of cystine to the diets of rats receiving bromophenol brought about a resumption of growth and increased the excretion of the sulphate ester whereas the addition of SO_4^{2-} ions to the diets was ineffective. It is therefore very likely that the SO_4^{2-} ions derived from cystine or cysteine by oxidative metabolism is the main source of sulphur available for conjugation.

Sulphate conjugation *in vitro* is a two-stage process

With the knowledge that sulphate ions are

involved in sulphate conjugation, Bernstein & McGilvery (1952a,b) studied the conversion of m-aminophenol to m-aminophenyl sulphate by a rat liver preparation. By differential centrifugation they obtained a fraction which provided the energy for the reaction and thereby the role of ATP (adenosine 5'-triphosphate) in sulphate conjugation was recognized. Bernstein and McGilvery (1952b) then proceeded to show that it was an active form of sulphate, and not of the phenol, that was produced by the reaction with ATP. By studying the kinetics of this system they were also able to deduce that at least two enzymes were involved in the sulphate-conjugating process. One of the enzyme systems required ATP and activated sulphate and the other simply participated in the reaction of the activated sulphate with the acceptor phenol to produce the sulphate ester.

Simultaneously, DeMeio & Tkacz (1952) were investigating the formation of phenyl sulphate by a liver homogenate and DeMeio, Wizerkaniuk and Fabiani (1953) were also able to show that the sulphate activating and transferring enzymes could be separated. Later work by DeMeio, Wizerkaniuk & Schreibman (1955) and by Segal (1955) very definitely separated the biosynthesis of sulphate conjugates into two distinct

and major steps. The first step is the activation of sulphate which is catalyzed by the sulphate activating enzymes. The second step then involves the transfer of the sulphuryl group from the active sulphate to the acceptor, the reaction being catalyzed by appropriate sulphate transferring enzymes grouped under the general name "sulphotransferases", formerly "sulphokinases". According to Enzyme Commission's recommendation "sulphotransferases" is the preferred nomenclature.

Sulphate conjugation is therefore a two-stage process consisting of a sulphate activating stage and a sulphate transferring stage.

The activation of sulphate ions

Hilz & Lipmann (1955) were the first to attempt to isolate the active sulphate. They showed that the incubation of ATP with radioactive sulphate in the presence of an ammonium sulphate fraction of a liver homogenate produced a labelled compound which could transfer its sulphuryl group to p-nitrophenol. From the stoichiometry of the reaction it was deduced that inorganic pyrophosphate was formed from ATP and since the active compound had an absorption peak at 260 mμ it was thought to be an adenylyl sulphate. Following

this work Robbins & Lipmann (1956b, 1957) characterized the active sulphate as 3'-phosphoadenosine-5'-phosphosulphate (PAPS), that is as 3'-phosphoadenylyl sulphate. The structure proposed by Robbins & Lipmann (1956b, 1957) was confirmed by the chemical synthesis of the active sulphate by Baddiley, Buchanan & Letters (1957).

The biosynthesis of PAPS is itself not a one-step enzymatic process as Wilson & Bandurski (1956) and Robbins & Lipmann (1956a,b, and 1958a) were able to show. They showed that the reactions can be represented by the following sequence

$$(1) \quad ATP + SO_4^{2-} \rightleftharpoons APS + PP$$

$$(2) \quad APS + ATP \longrightarrow PAPS + ADP$$

$$\text{Adding, } 2ATP + SO_4^{2-} \longrightarrow PAPS + ADP + PP$$

Where APS is adenosine 5'-phosphosulphate, PP is inorganic pyrophosphate and ADP is adenosine 5'-diphosphate. Reaction 1 is catalyzed by ATP-sulphurylase(ATP : sulphate adenylyltransferase, EC 2.7.7.4) and is characterized by its extremely unfavourable equilibrium for APS formation. At pH 7.8 Robbins & Lipmann (1958b) found an apparent equilibrium constant, K', of 10^{-8} where

$$K' = \frac{[APS]\ [PP]}{[ATP]\ [SO_4^{2-}]}$$

so that the standard free energy change for the reaction is +11,000 calories. The forward reaction is therefore thermodynamically extremely difficult to proceed; but fortunately reaction (2), which is catalyzed by APS - phosphotransferase (ATP : adenylylsulphate, 3'-phosphotransferase 2.7.1.25) is practically irreversible with a standard free energy change of -5,000 calories and so it is reaction (2) that drives the entire reaction in the forward direction and thereby allows PAPS to accumulate.

ATP-sulphurylase is absolutely specific for ATP (Robbins & Lipmann, 1958a; Wilson & Bandurski, 1958; Akagi & Campbell, 1962), but can utilize other Group VI anions, namely SeO_4^{2-}, WO_4^{2-}, MoO_4^{2-} and CrO_4^{2-}. APS-phosphotransferase, on the other hand, is not specific for ATP since other nucleoside triphosphates are substrates (Robbins & Lipmann, 1958a). The specificity for APS also is not absolute as Wilson & Bandurski (1958) found that APSe (adenosine 5'-phosphoselenate), obtained from reaction (1) above by replacing SO_4^{2-} ions by SeO_4^{2-} ions, could presumably act as a substrate.

Vitamin A in sulphate activation

It was shown by Wolf & Varandani (1960) that the incorporation of $^{35}SO_4$ ions and ^{14}C-glucose into mucopolysaccharides *in vitro* by rat colonic segments was greatly depressed during vitamin A deficiency. The activity of the preparations from such deficient rats could be restored to normal by the addition of retinol, retinal or retinoic acid *in vitro*. Further study of preparations from pig colonic mucosa localized this defect to the reduction of PAPS synthesizing efficiency (Varandani, Wolf & Johnson, 1960) during vitamin A deficiency. This work was essentially confirmed by the results of SubbaRao, Sastry & Ganguly (1963) and SubbaRao & Ganguly (1964) who showed that the ability to form PAPS was greatly reduced in the liver and colon of vitamin A deficient rats. This activity could be restored to normal by the addition of retinol or retinoic acid *in vitro*. Controversy arose when Pasternak, Humphries & Pirie (1963) could not detect any change in the sulphate activating ability of the colonic mucosa or corneal epithelium of vitamin A deficient rats or rabbits. This discrepancy was however explained by SubbaRao and Ganguly (1964) who pointed out that the criteria adopted by Pasternak,

Humphries & Pirie (1963) were not adequate to presuppose total vitamin A deficiency in their experimental animals. On the other hand, Hall & Straatsma (1966) have shown that in animals deficient in vitamin A by the criteria of SubbaRao & Ganguly (1964), the synthesis of PAPS in the liver is decreased whereas that in the retina is increased.

A very recent work by Sundaresan (1966) seems to have attributed the decrease in the formation of PAPS during vitamin A deficiency to a decrease in the ATP-sulphurylase activity in rat liver and colon. This decrease in the ATP-sulphurylase activity of the preparation from deficient animals cannot be overcome by the addition of retinol or retinoic acid <u>in vitro</u>. On the other hand the activity of the preparations from deficient animals can be restored to normal by the addition of an acidic lipid factor prepared from the ATP-sulphurylase of normal animals by butanol extraction at pH 5. This factor is not identical with retinol or retinoic acid as shown by chromatography. Although Sundaresan's work (1966) clearly implicates a vitamin A type cofactor for mammalian ATP-sulphurylase activity, no such cofactor is necessary for microbial ATP-sulphurylase. This difference in the properties of an important enzyme from two sources seem to be unrealistic.

Obviously much work is needed in this field and the isolation of a homogeneous ATP-sulphurylase from a mammalian source will seem to be required for a definitive answer on the role of vitamin A in sulphate activation.

The transfer of sulphate group

As discussed earlier, the final stage of sulphate conjugation *in vivo* involves the transfer of the sulphuryl group from PAPS, the donor, to the appropriate acceptor and this transfer is catalyzed by a group of enzymes called the sulphotransferases. As an example, the transfer of the sulphuryl group from PAPS to p-nitrophenol is catalyzed by phenol sulphotransferase (3'-phosphoadenylyl sulphate : phenol sulphotransferase, EC 2.8.2.1).

A number of different types of methods are available for the determination of the sulphotransferases. The most general method is based on the use of PAPS labelled with ^{35}S and then utilizes chromatographic separation (Vestermark & Boström, 1959; Spencer, 1960); but a most valuable method is that of Wengle (1964a) which utilizes the fact that the barium salts of SO_4^{2-} ions are insoluble whereas the barium salts of sulphate

esters are generally soluble. Another fairly general and extremely useful method is based on the solubility in chloroform of the methylene blue salts of many weakly polar sulphate conjugates (Roy, 1956a). The method cannot be used to determine the sulphate esters of simple alcohols or carbohydrates but has proved most valuable in the study of aryl and steroid sulphates and aryl sulphamates.

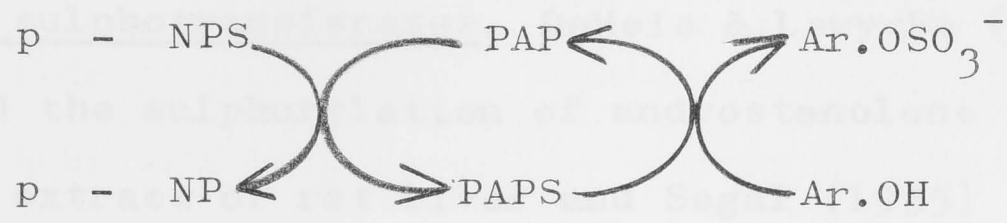
There are other specific methods for the determination of p-nitrophenyl sulphate and m-aminophenyl sulphate but their use is obviously restricted.

Phenol sulphotransferase. The phenol sulphotransferases appear to be most studied of all the sulphotransferases and are among the most widespread of the sulphotransferases and they seem to occur in almost all mammalian tissues (Holcenberg & Rosen, 1965; Boström & Wengle, 1965). The reaction catalyzed by phenol sulphotransferase can be represented by

$$\text{Ar. OH} + \text{PAPS} \rightleftharpoons \text{Ar. OSO}_3^- + \text{PAP}$$

Where Ar is the aromatic nucleus and PAP is adenosine-3', 5'-diphosphate. The reaction is reversible if the 'sulphate group potential' of the aryl sulphate is high as in the case of p-nitrophenyl sulphate or 3,5-dinitrophenyl sulphate (Gregory & Lipmann, 1957). The reverse reaction forming p-nitrophenol (p-NP) and

PAPS from p-nitrophenyl sulphate (p-NPS) and PAP under the catalytic influence of a partially purified phenol sulphotransferase from rabbit liver has been studied by Gregory & Lipmann (1957) and Brunngraber (1958). They showed that if the reaction is carried out in the presence of a phenol whose sulphate has a group potential less than that of p-nitrophenyl sulphate then the sulphuryl group of p-nitrophenyl sulphate is transferred to the phenol forming its sulphate. Other types of potential acceptors, such as steroids are inactive in this system. It must be presumed that PAPS is formed as an intermediary and the reactions can be represented by



PAPS must remain enzyme-bound otherwise transfer to nonphenolic acceptors would have been expected. A recent finding by Wortman (1961) that the mucopolysaccharide sulphotransferase of beef cornea can utilize p-nitrophenyl sulphate as the sulphate donor in the presence of PAP, presumably through a coupling of a phenol sulphotransferase acting in the reverse direction, suggests that PAPS must dissociate from this

phenol sulphotransferase or else that the enzyme exhibits multiple specificity, though it is unlikely. Hence the transfer reaction first studied by Gregory & Lipmann (1957) may not be regarded as a general reaction for all phenol sulphotransferases.

There is still considerable doubt as to the number of phenol sulphotransferases present in the mammalian tissues and the only direct observations bearing on this problem are those of Banerjee & Roy (1966a) who, by chromatography on DEAE-Sephadex, have separated from guinea pig liver extracts three zones showing phenol sulphotransferase activity. This work is discussed later.

Steroid sulphotransferases. DeMeio & Lewycka (1955) first observed the sulphurylation of androstenolone in a soluble extract of rat liver and Segal (1955) detected oestrone sulphate in a similar system. Out of 32 steroids tested in a particle-free rabbit liver extract, Schneider & Lewbart (1956) found that 14 were sulphurylated. Further work has been done with rat liver fractions by Roy (1956b), with rat and beef liver by DeMeio, Lewycka, Wizerkaniuk & Salciunas (1958) and in frog liver homogenates by Bridgwater & Ryan (1957). There is still considerable doubt as to

how many separate steroid sulphotransferases are present and the first direct work concerning this point is that of Nose & Lipmann (1958) who by electrophoresis showed that the enzymes responsible for the production of androstenolone sulphate and oestrone sulphate are different. The sulphurylating activities towards androstenolone, androsterone and pregnenolone were, however, superimposable after electrophoresis.

With regard to oestrone sulphotransferase the only highly purified enzyme which has been investigated is that from ox adrenal (Adams, 1967). This is an SH enzyme which is activated by divalent cations (Mg^{2+}, Ca^{2+} and Mn^{2+}) and which is reported as being specific for oestrogens having no action on simple phenols such as phenol or p-nitrophenol. The more complex phenols stilboestrol and hexoestrol are slowly sulphated so that the specificity of the enzyme for steroid nucleus cannot be absolute.

Arylamine sulphotransferase. The activity ascribed to it was first described by Roy (1960a) in rat and guinea pig liver which catalyzed the formation of 2-naphthyl sulphamate, a metabolite of 2-naphthylamine (Boyland, Manson & Orr, 1957). This reaction is rather different from the other sulphate conjugations because an N-sulphate was produced from an aromatic amine as

shown below.:-

$$\underset{\text{(arylamine)}}{Ar.\,NH_2} + PAPS \longrightarrow \underset{\text{(aryl sulphamate)}}{Ar.NH.SO_3^-} + PAP$$

Interest in this enzyme was stimulated by the fact that 17-oxosteroids had a pronounced effect on the reaction acting as partially competitive inhibitors for the guinea pig enzyme and activators for the rat enzyme (Roy, 1961, 1962, 1964).

Choline sulphotransferase. This enzyme catalyzes the transfer of the sulphuryl group from PAPS to choline with the formation of choline O-sulphate. The choline sulphotransferase is of restricted occurrence and is formed only in certain higher fungi (Harada & Spencer, 1960; Spencer & Harada, 1960) that can utilize choline O-sulphate as a store of sulphur. It was at one time claimed that APS was the sulphuryl donor for the choline sulphotransferase reaction (Kaji & McElroy, 1958), but it has now been shown that the enzyme, like all the other sulphotransferases, can only utilize PAPS as sulphate donor (Kaji & Gregory, 1959). The reaction is irreversible.

Mucopolysaccharide sulphotransferase. This group of sulphotransferases is probably the largest but the least understood because none of the enzymes have been

appreciably purified. The enzymes seem to be widespread in tissues containing mucopolysaccharides - that is, in most tissues of the body. All the mucopolysaccharide sulphotransferases catalyze the transfer of the sulphuryl group to the appropriate hydroxyl group of the sugar residues; for example, in the chondroitin sulphates and the heparitins the sulphuryl group is transferred to the various hydroxyl groups in the hexosamine portion of the molecule. In the synthesis of heparin an additional reaction, namely the transfer of sulphuryl group to the amino group of the glucosamine residues must also take place. That at least two sulphotransferases are involved in the biosynthesis of heparin has clearly been shown by Korn (1959a,b) who found that slices of mast cell tumour incorporated $^{35}SO_4$ ions into heparin with approximately equal amounts appearing as O-sulphate and N-sulphate groups whereas particle free extracts of the tumour catalyzed the formation of O-sulphate groups only.

In all the mucopolysaccharides one problem of special interest is the nature of the physiological acceptors for the enzymes. In vitro these acceptors can range from preformed polysaccharides through to mono-saccharides. Recent evidence puts preformed polysaccharides as the true acceptors in vivo. Suzuki &

Strominger (1959) found no incorporation of uridine diphosphoacetylgalactosamine sulphate into mucopolysaccharide in extracts of the isthmus regions of hen oviduct, where the incorporation from PAPS is known. The uridine compound is a potential intermediate normally present in the tissue.

Vitamin A in sulphate transfer

SubbaRao, Sastry & Ganguly (1963) and SubbaRao & Ganguly (1964) were the first to show that the level of phenol sulphotransferase activity was low in the extracts from the livers of rats deficient in vitamin A. The activity could be restored to normal by adding retinol or retinoic acid *in vitro*.

Carroll & Spencer (1965a) showed that in the livers of foetal rats the activities of all the sulphotransferases were much lower than the normal adult values. However the activities increased rapidly after birth and usually after 7 days reached the normal adult values. Carroll & Spencer (1965b) attributed this reduction in sulphotransferase activity to the physiological deficiency of vitamin A since the addition of retinol or retinoic acid *in vitro* restored the transferase activities to adult values. These

observations led Carroll & Spencer (1965b) to suggest that the foetal rat livers contain sulphotransferases in inactive forms which are activated only after birth by the ingestion of vitamin A from milk. This vitamin A effect in foetal liver is not universal, as Spencer & Raftery (1966) found the effect to be absent in foetal guinea pig liver which may of course have been replete with vitamin A and also as Wengle (1964b) did not observe the effect in human foetal liver.

At present, then, the evidence that vitamin A, or perhaps a metabolite of vitamin A, is required by the sulphotransferases is only circumstantial. Once again an unambiguous solution to the problem will lie in the isolation of the suspected lipid factor from highly purified sulphotransferases.

Other routes for sulphate conjugation

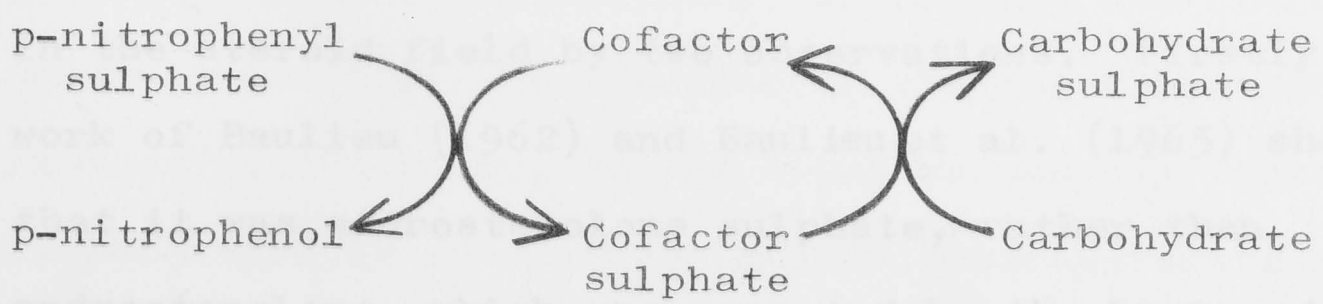
As seen above the major route for sulphate conjugation *in vivo*, as *in vitro*, involves the transfer of the sulphuryl group from PAPS and so ultimately involves the utilization of SO_4^{2-} ions. However two other pathways have been reported. The first of these implies a direct utilization of the sulphur of cysteine, without prior conversion of this to

SO_4^{2-} ions (Wellers, 1954). The mechanism proposed by Wellers (1954) for this conjugation envisages the formation of C-O bonds from C-S bonds and thus involves considerable chemical difficulty. In recent years Wellers & Boelle (1960) have carried out some very accurate sulphur balance studies on rats fed artificial diets of known sulphur content and have claimed that the results support the above ideas. Their results showed that when methionine was used as the sole sulphur source in the diet there was a positive sulphur balance (that is, less sulphur was excreted than was ingested) and that this was increased by adding indole to the diet. The increase in the sulphate ester content of the urine consequent to the administration of indole was less than the increase in the sulphur balance. When cysteine was used as the sole sulphur source in the diet, there was a slightly negative sulphur balance which was decreased on simultaneously administering indole. In this case the resultant increase in the sulphate ester content of the urine was much greater than the change in the sulphur balance. Finally when sodium sulphate was used as the sole dietary source of sulphur there was a highly negative sulphur balance which was increased by adding indole to the diet. The increase in the negativity of the

sulphur balance was equal to the increase in the sulphate ester content of the urine caused by indole ingestion. The above results were interpreted by Wellers & Boelle (1960) as being sufficient evidence to show that cysteine is a very efficient source of sulphur for conjugation but SO_4^{2-} ions are not utilized. These conclusions of Wellers & Boelle (1960) are not fully justified since their results only show that dietary SO_4^{2-} ions are not utilized for sulphate conjugation and may merely confirm that SO_4^{2-} ions are only poorly absorbed from the gastrointestinal tract. At this point one recalls that Bray, Humphris, Thorpe, White & Wood (1952) showed that, at least in the rabbit, the level of endogenous SO_4^{2-} ions is normally rate limiting in the formation of aryl sulphates - one more piece of evidence in favour of SO_4^{2-} ions as being the source of sulphur for conjugation.

The other pathway involves a transfer of the sulphuryl group from p-nitrophenyl sulphate to carbohydrates utilizing the reverse reaction by an arylsulphatase. This possibility emerged from the work of Suzuki, Takahashi & Egami (1957) who showed that acetone-dried preparations of the mucous gland of the mollusc, Charonia lampas, could not incorporate $^{35}SO_4^{2-}$ into charonin sulphate (a glucan polysulphate

containing both amylose and cellulose structure); but could so incorporate the sulphate group of p-nitrophenyl ^{35}S-sulphate. These workers attributed this transfer reaction - in analogy to the transfer reactions of phosphate catalyzed by phosphatase (Axelrod, 1956) - to the participation of arylsulphatase present in the enzyme preparation; since, like the purely hydrolytic reaction catalyzed by the arylsulphatases this transsulphurylation reaction was inhibited by F^- and PO_4^{3-} ions. Subsequently it was shown by Suzuki, Takahashi & Egami (1959) that partly purified sulphatases of the mucous gland could not bring about the transfer but partly purified preparations from the digestive gland could do so provided that the preparations of charonin sulphate used as acceptor were not highly purified. It was later concluded that relatively crude preparations of charonin sulphate contained a dialysable cofactor which was essential for the transfer reaction and the scheme of reaction as represented by Egami & Takahashi (1962) is



Although the scheme outlined above seems attractive, a few details are missing, namely that the implication of arylsulphatase is assumed simply on the basis of inhibition by F^- and PO_4^{3-} ions and that the nature of the cofactor is unknown. Be that as it may, the biochemical significance of the transfer is hard to judge since p-nitrophenyl sulphate is not known to be a normal metabolite and is very reactive owing to a high sulphate group potential.

The revival of interest in sulphate esters

Although it has long been known from studies *in vivo* that most animals can synthesize many different types of sulphate conjugates (R.T. Williams, 1959) and that sulphate conjugation in various mammalian tissues has been the scope of many investigations (Lipmann, 1958; Roy, 1960b; Gregory & Robbins, 1960; Boström & Vestermark, 1961), the sulphate conjugates had hitherto been regarded as metabolic end products destined for excretion. This view has greatly been changed at least in the steroid field by two observations. Firstly, the work of Baulieu (1962) and Baulieu et al. (1965) showed that it was androstenolone sulphate, rather than androstenolone, which was secreted by the human adrenal

gland and that androstenolone sulphate can be further metabolized to the oestrogens during pregnancy (Baulieu & Dray, 1963). The synthesis of steroid sulphates by human adrenal gland was also noted by Wallace & Lieberman (1963); by Cohn, Mulrow & Dunn (1963) and by Adams (1963) and its secretion from the human adrenal gland by Wieland et al. (1963). Secondly, the work of Roberts et al. (1964a,b); Calvin, VandeWiele & Lieberman (1963) and Calvin & Lieberman (1964) clearly showed that cholesteryl sulphate could be converted in vivo to androstenolone sulphate without the fission of the sulphate ester linkage. All these observations therefore opened up the possibility that sulphate esters could act as substrates for hormone biosynthesis and take part in normal metabolic inter-conversions.

With this revival of interest in the sulphate esters it seemed important to reinvestigate in greater detail the enzymatic mechanisms involved in their formation. As has been seen earlier the actual incorporation of the sulphuryl group into the acceptor molecule involves the transfer of the sulphuryl group from PAPS, the donor, to the acceptor and the reaction is catalyzed by the appropriate sulphotransferase. Although it is well known that the sulphotransferases

are widely distributed in mammalian tissues, both endocrine and otherwise, there is considerable doubt as to their specificity or their number in, for instance, mammalian liver which is a very active tissue for the sulphate conjugation of various types of organic compounds, namely phenols, phenolic steroids, steroid alcohols, arylamines and aliphatic alcohols. As seen before, Nose & Lipmann (1958) partially separated the sulphotransferases of rat liver and were able to show the separate identities of the enzymes responsible for the production of androstenolone sulphate, oestrone sulphate and p-nitrophenyl sulphate. These were really all the data that gave a direct indication of the number of sulphotransferases available for sulphate conjugation.

Meanwhile, very similar reactions catalyzed by the phosphotransferases which are involved in the transfer of the phosphate group from the donor, adenosine 5'-triphosphate (ATP), to a suitable acceptor were being extensively studied by several workers. The kinetic studies on three such phosphotransferases have shown that these enzymes act by a rapid equilibrium randon mechanism after Cleland (1963a) in which the interconversion of the central ternary complexes is the slowest and hence the rate controlling steps

(Reynard, Hass, Jacobsen & Boyer, 1961; Fromm & Zewe, 1962; Morrison & James, 1965). These kinetic studies therefore posed the question whether the sulphotransferase reactions had similar mechanisms.

The present work therefore began as a reinvestigation of the sulphotransferases of guinea pig liver. This species was chosen because of a relatively high rate of aryl sulphamate synthesis by its liver and particularly since this activity was strongly inhibited by low concentrations of 17-oxosteroids which apparently combined with the enzyme through the D ring (Roy, 1960a, 1961) - an effect which was later suggested by Roy (1964) to be allosteric and so of inherent interest in the light of the work of Monod, Changeux & Jacob (1963). Liver was chosen simply because it contains almost all types of sulphotransferases, is abundant and easy to process.

The first stage of the present work began with the isolation and purification of the sulphotransferases in the hope of elucidating the role of vitamin A in sulphate conjugation and to study the mechanisms by which the sulphotransferase reactions proceed. By methods already described by Banerjee & Roy (1966a), it was possible to obtain, for the first time, a specific phenol sulphotransferase free from

any steroid and arylamine sulphotransferase activity. This enzyme was therefore chosen as the model for kinetic studies in detail and the work is the starting point for the elucidation of sulphotransferase reaction mechanisms.

CHAPTER 2

MATERIALS AND METHODS

Preparation and Analysis of 3'-Phosphoadenylyl Sulphate (PAPS)

A. Preparation

Crude PAPS was prepared using the sulphate activating enzymes of rat liver. The sulphate activating enzymes were prepared as follows. The liver was homogenized with 3 volumes of 0.15M KCl - 1 mM EDTA (pH 7) in an Omnimixer (Ivan Sorvall Inc., Conn., U.S.A.). After centrifuging, the supernatant was treated with saturated $(NH_4)_2SO_4$ (60 ml $(NH_4)_2SO_4$ per 100 ml supernatant). The precipitate was rejected and the supernatant was treated with 75 ml saturated $(NH_4)_2SO_4$ per 100 ml of the original supernatant. The precipitate was dissolved in water so that 1 ml of the resulting solution contained 500 mg of liver. This gave an enzyme fraction precipitating between the approximate limits of 1.5 and 2.3M $(NH_4)_2SO_4$ (Roy, 1960a). This enzyme fraction was incubated as previously described by Roy (1960a) except that the concentration of ATP was increased to 0.014M and the

time of incubation to $1\frac{1}{2}$ hrs. Proteins were precipitated from the reaction mixture by adding 3 volumes of ethanol. The precipitated proteins were centrifuged off and the supernatant concentrated to about 15 ml in a rotary vacuum evaporator at a water bath temperature of 37^{o}. The pH of the concentrate was adjusted to 7.2 with 1N NaOH and the solution clarified by filtration. The PAPS concentrate was then applied to a column (1.5 x 18 cm) of Dowex 1 x 10 (Cl^- form). Being more acidic PAPS is much more strongly adsorbed to the Dowex anion exchanger than ATP. The column was washed with water and then with 0.5M NaCl until the absorbance at 260 mμ had fallen below 0.030, the latter eluting all unreacted ATP. Elution was then commenced with 1M NaCl which eluted PAPS (Brunngraber, 1958). The fractions containing PAPS were combined (usually 700 ml) and stirred with acid-washed Norit-A charcoal for 10 minutes to adsorb the nucleotide. 1g of the activated charcoal was used for every 3μ moles of PAPS based on the absorbance at 260 mμ assuming a molar absorption coefficient of 15×10^3 at 260 mμ. The charcoal was then washed on the filter with water to remove Cl^- ions. PAPS was eluted from the charcoal with about 1 litre of aqueous ethanol (50% $^{v}/_{v}$) containing 1% of

1M NH_4OH. The PAPS eluate was concentrated in a rotary vacuum evaporator as before to about 25ml to remove all ethanol. The pH of the solution was adjusted to 7.2 and, after filtration its volume was adjusted with water to make the PAPS concentration 0.33 mM based on the absorbance of the solution at 260 mμ. Frequently it was necessary to freeze the solution before filtration through a sintered glass funnel to remove very fine particles of charcoal. The solution was then stored at -30^{o}.

The usual yield of PAPS was about 1.5 micromole per g of liver, amounting to about 2.5% of the ATP used in the incubation mixture.

B. Analysis

Determination of PO_4^{3-}

Phosphate was determined by the method of Fiske & Subbarow (1925). The principle of the method is that PO_4^{3-} ions are converted to phosphomolybdic acid in the presence of H_2SO_4. The phosphomolybdic acid is then reduced by 1-amino-2-naphthol-4-sulphonic acid to give molybdenum blue. The absorbance of the resultant solution is measured at 660 mμ. The amount of PO_4^{3-} is then determined from calibration curves

obtained by using standard phosphate solutions.

Labile PO_4^{3-}: The 3'-phosphate group of PAPS is labile and can be easily hydrolyzed by acid (Robbins & Lipmann, 1957). The labile phosphate of PAPS was determined by heating 1 ml of PAPS (0.33 mM) in a water bath at 100° with 1 ml of 2N HCl for ½ hr. The mixture was then neutralized with 2 ml of 1N NaOH. To the solution was added 0.20 ml of 10N H_2SO_4 and after mixing, 0.40 ml of 2.5% ammonium molybdate solution was added and mixed. Finally 0.20 ml of a freshly prepared and filtered reducer solution (containing 0.5 g 1-amino-2-naphthol-4-sulphonic acid in 195 ml of 15% $NaHSO_3$ and about 7 ml of 20% Na_2SO_3) was added to the reaction mixture and the total volume made up to 5 ml with water. After thorough mixing, the solution was allowed to stand for 10 min and the absorbance at 660 mμ was measured (Fiske & Subbarow, 1925).

Total PO_4^{3-}: 0.5 ml of PAPS was digested with 0.2 ml 10N H_2SO_4 on electrically controlled heaters for 1 hr. When all the water escaped and the very faint brownish colour disappeared, the solution was cooled and 0.5 ml of water was added. The sample was then heated at 100° on a water bath for 10 minutes to

decompose pyrophosphate. The mixture was quantitatively transferred to a 5 ml measuring flask by repeated washings with water so that the total volume was kept to 4 ml. Then 0.40 ml of the ammonium molybdate solution was added and, after mixing, 0.20 ml of the reducer was added to the mixture and then the volume was made up to 5 ml with water. The reaction mixture was shaken thoroughly and was allowed to stand for 10 minutes and, as before, the absorbance at 660 mμ was measured (Cowgill & Pardee, 1957).

Determination of transferrable sulphate.

Transferrable sulphate was determined by incubating known concentrations of PAPS and 2-naphthol with a large excess of phenol sulphotransferase. 2-naphthol was chosen as acceptor because the formation of 2-naphthyl sulphate by the sulphurylation of 2-naphthol is essentially irreversible. The ratio of the amount of total transfer effected to the total PAPS used gave the transferrable sulphate.

Analytical data for PAPS.

Analytical results from several batches of PAPS are given below. The adenosine content was obtained from the absorbance at 260 mμ assuming a

molar absorption coefficient of 15×10^3.

Batch	Adenosine content	Labile PO_4^{3-}	Total PO_4^{3-}	Transferrable SO_4^{2-}
1	1.00	0.98	1.95	0.82
2	1.00	1.00	1.94	0.79
3	1.00	1.01	1.96	0.83
4	1.00	1.02	1.90	0.85
5	1.00	-	-	0.85

The experimental values for labile and total phosphate are in good agreement with the theoretical values. The transferrable sulphate was, however, never more than 0.85. Although this value may seem a bit low, it compares very favourably with the transferrable sulphate values (0.20 - 0.85) given by Robbins & Lipmann (1957). The method of preparation of PAPS therefore gave a product of precise composition.

Determination of Sulphate Esters

Preparation of phenyl sulphate

Phenyl sulphate was prepared by the general method of preparation of aryl sulphates using dimethylaniline-SO_3 complex (Gilbert, 1962). 1 mole of

dimethylaniline (dried over KOH pellets) was taken up in 150 ml of dry chloroform (dried over $CaCl_2$ and filtered) in a flask cooled to 0^o and 26 ml (0.4 mole) of redistilled chlorosulphonic acid was added dropwise with constant stirring under dry conditions. The temperature was kept below 10^o. Then 28g (0.3 mole) phenol in dry chloroform was added with stirring to the dimethylaniline-SO_3 complex formed in the reaction flask. The mixture was stirred at 0^o for 1 hr. It was then allowed to stand overnight at room temperature. The yellow precipitate of dimethylaniline-SO_3 complex, which had formed, gradually disappeared by reacting with phenol.

The solution was then poured onto 68g KOH in approximately 500 ml of water. The mixture was shaken thoroughly in a separating funnel. The organic layer at the bottom was discarded and the aqueous layer was concentrated in a rotary vacuum evaporator on a boiling water bath. Concentration was continued till heavy crystallization ensued. The mixture was cooled in a refrigerator and the crystals were filtered under vacuum and washed with the minimum quantity of cold water. The crystals were then dissolved in the minimum of boiling water and the solution was allowed to cool to crystallize. The crystals were again washed with

cold water to remove Cl^- and SO_4^{2-} ions and the phenyl sulphate was recrystallized from hot water. The phenyl sulphate crystals were then dried over P_2O_5 in a vacuum desiccator.

Sulphate esters of p-nitrophenol and other phenols, steroids and 2-naphthylamine (the sulphamate) which were gifts from Dr. A.B. Roy were used for calibration by the methylene blue technique. p-Nitrophenyl sulphate was crystallized several times to remove all free SO_4^{2-} ions and p-nitrophenol. (p-Nitrophenyl sulphate is rather labile and can undergo autocatalytic hydrolysis to p-nitrophenol and SO_4^{2-} ions during recrystallizations).

Determination of sulphotransferases.

The method was based on that previously described by Roy (1956a) for the determination of steroid sulphates but differed in using PAPS as the sulphuryl donor. The method utilizes the extraction of the methylene blue salts of weakly polar sulphate conjugates from aqueous medium by chloroform. The method is extremely useful in the determination of aryl and steroid sulphates where it is equally highly sensitive as will be seen below, and in the

determination of aryl sulphamates where the sensitivity is lowered because of the higher polarity of the sulphamates. The method cannot be used to determine the highly polar sulphate esters of simple alcohols or carbohydrates.

The reaction mixtures usually had the composition given in Table 1 and after incubation of these at 37^{o} for the appropriate time, the reaction was stopped by the addition of 1 ml of methylene blue reagent (Roy, 1956a). 5 ml of chloroform was added to the mixture and after vigorous shaking the mixture was briefly centrifuged. The chloroform layer was separated and dried over Na_2SO_4. The absorbance of the solution was measured at 650 mμ in a Unicam S.P. 600 spectrophotometer. The amount of sulphate esters produced were then obtained from suitable calibration curves. The calibration curves were linear up to at least 100 millimicromoles of sulphate conjugates in the reaction mixtures. In the case of p-nitrophenyl sulphate, Lambert-Beer Law was obeyed up to an absorbance of 1.2.

The amount of various sulphate conjugates giving an absorbance of 0.100 are tabulated below:-

Sulphate conjugate	Millimicromoles of sulphate conjugate in 1 ml reaction mixture to give an absorbance of 0.100.
p-Nitrophenyl sulphate	6.25
1-Naphthyl sulphate	6.30
2-Naphthyl sulphate	6.63
Phenyl sulphate	20.5
2-Naphthyl sulphamate	30.7
Androstenolone sulphate	6.08

The assay of phenol sulphotransferase using p-nitrophenol as acceptor was usually carried out using the methylene blue technique. Another way to assay this reaction is to determine the disappearance of p-nitrophenol from the reaction mixture. The principle of the method is that p-nitrophenol (pK 7.2) is coloured yellow in the dissociated form. Obviously the formation of the sulphate ester eliminates the hydroxyl group and so prevents the formation of the coloured nitrophenoxide anion, hence by measuring the diminution of the absorbance at 400 mμ, the amount of nitrophenol reacted can be determined. This latter method has been used in the measurement of the trans-sulphurylation reactions as will be seen later.

The methylene blue value for p-nitrophenyl

sulphate was then compared with the disappearance of p-nitrophenol obtained by the spectrophotometric experiment. For this parallel enzymatic assays were carried out using the two methods. The composition of the incubation medium was 0.1M Tris-acetic acid buffer (pH 7.5), 0.100 mM p-nitrophenol, 0.100 mM PAPS and a fixed amount of phenol sulphotransferase. Incubation was for 10 min. The value of p-nitrophenyl sulphate formed (methylene blue value) and the p-nitrophenol disappeared (spectrophotometric value) at two different enzyme concentrations are tabulated below.:-

Experiment	p-nitrophenyl sulphate formed (methylene blue value), milli-micromoles/10 min.	p-nitrophenol disappeared (spectrophotometric value) milli-micromoles/10 min.
1	13.8	13.6
2	17.2	17.3

The two methods therefore give essentially equal results.

Table 1

Experimental conditions for sulphotransferase assays

The concentrations given in the table are the final ones in the reaction mixtures, which had a volume of 1 ml. The soluble phenols and 2-naphthylamine (as hydrochloride) were added in aqueous solution while the steroids were added in 0.1 ml of a 1 mM solution in propylene glycol.

	Phenol sulphotransferase	Steroid sulphotransferases		
Acceptor substrate	p-Nitrophenol	Steroid	p-Nitrophenol	2-Naphthylamine
Concn (mM)	1.0	0.1	1.0	2.0
PAPS concn. (mM)	0.1	0.1	0.1	0.1
Buffer type	Acetate, pH 5.8	Tris, pH 7.5	Tris, pH 7.5	Tris, pH 7.5
concn (M)	0.1	0.1	0.1	0.1
Mg^{2+} concn (mM)	0	2 mM excess over EDTA in enzyme		
Reaction time (min)	30	30	30	30

The recoveries of the various sulphate esters added to the appropriate reaction mixture containing 0.6 mg protein per ml were as follows : 23.8 and 47.5µm moles p-nitrophenyl sulphate, 100% recovery; 8.6 and 25.3 µm moles androstenolone sulphate, 97% recovery; 68.4 and 135 µm moles 2-naphthyl sulphamate, 98% and 100% recovery. For the present purpose these recoveries could all be regarded as quantitative.

In the case of cholesteryl sulphate which is firmly bound to protein (Roy, 1963) the above method gave a recovery of only 40% from a reaction mixture containing 2 mg/ml of protein. The following altered procedure (Roy, 1956b) was therefore employed to determine cholesteryl sulphate. After incubation at 37° for the appropriate time the reaction was stopped by adding 5 ml of ethanol. Precipitated proteins were centrifuged off and a 5 ml sample of the supernatant was evaporated on a boiling water bath for 10 min., leaving a small residue of propylene glycol which, after cooling was mixed with 2 ml of methylene blue reagent (diluted 1:1 with water) and extracted with chloroform. The amount of cholesteryl sulphate was determined as above. With this altered method of assay the recovery of cholesteryl sulphate was increased to

99% from 40% (Banerjee & Roy, 1967).

Techniques of Protein Fractionation

Acetone fractionation

Acetone fractionation of the enzyme was carried out at temperatures ranging from -5^{o} to -10^{o}. To the enzyme preparation containing 0.003M $CaCl_2$ the requisite amount of acetone was added dropwise and the rate of addition was adjusted so that the enzyme mixture always remained at the temperature range -5^{o} to -10^{o} (Askonas, 1951). With high proportions of acetone in the mixture, the latter could be kept at -10^{o} without freezing. The acetone precipitated mixtures were then kept in the low temperature bath for $\frac{1}{2}$ hr. and then centrifuged at the same temperature. The precipitates were then dissolved in 0.01M Tris-acetic acid buffer, pH 7.5, containing 0.01M mercaptoethanol and the solutions were dialysed against many changes of the same buffer to remove all the acetone.

The following acetone concentrations were used for precipitation: 16%, 20%, 27%, 33%, 43%, 50% and 60% $^{v}/_{v}$.

$(NH_4)_2SO_4$ Fractionation

Saturated solutions of $(NH_4)_2SO_4$ were prepared as follows. An excess of 'Analar' $(NH_4)_2SO_4$ was dissolved in hot 0.01M EDTA-NaOH buffer, pH 7.5, and the pH was readjusted to 7.5 with NaOH after the solution had cooled to room temperature. The solution was filtered and the saturated ammonium sulphate was then stored in a refrigerator at 4^o. On cooling a saturated ammonium sulphate solution at 4^o was obtained.

This saturated ammonium sulphate solution was used for finer precipitations of the sulphotransferases. Assuming no contraction in volume when saturated ammonium sulphate was added to the enzyme solution, the enzyme was differentially precipitated by successive additions of $(NH_4)_2SO_4$, that is after each addition of $(NH_4)_2SO_4$ the mixture was centrifuged and the supernatant was measured and precipitated at a higher $(NH_4)_2SO_4$ concentration by adding more $(NH_4)_2SO_4$. The precipitates from all the stages were dissolved in equal amounts of 0.01M EDTA NaOH buffer, pH 7.5, containing 0.01M mercaptoethanol. The solutions were then assayed to see if any purification could be achieved by the procedure. All the sulphotransferases

were precipitated together between 0.3 and 0.6 $(NH_4)_2SO_4$ saturation.

Preparation of Sephadex G-200 and G-100 columns

The dry Sephadex G-200 and G-100 powders were suspended in excess distilled water for at least 3 days. Repeated sedimentation and decantation were then carried out to remove the fines. The gels were then transferred into the equilibrating buffers. After several changes of the buffer by decantation, the gels were stored in their respective buffers.

The requisite amount of the gel was then poured into the chromatographic column in a steady stream taking care that no air bubbles remained entrapped in the mixture. When a layer of a few centimeters had formed at the bottom the column outlet was opened. The rest of the gel was then added and after the column was evenly packed, washing with buffer was continued till equilibration.

The column was charged by pumping the enzyme (frequently made denser by adding 0.1g sucrose per 10 ml) on top of the gel bed, protected with a stainless steel gauze, without removing the supernatant buffer. Being denser the enzyme formed a sharp layer between

the top of the gel and the eluant buffer. The column outlet was closed during charging. The outlet was opened when all the enzyme had been added to the column which was developed downward by gravity flow. Fractions were collected by means of a fraction collector.

Preparation of Sephadex anion exchanger, DEAE-Sephadex, column.

DEAE-Sephadex was allowed to swell in distilled water and the fines were removed by repeated sedimentation and decantation. The gel was then successively washed with 1M and 2M NaCl on Buchner funnel. The gel was then washed with water and then with 0.5N NaOH until the washings were free from Cl^- ions. The excess of NaOH was removed by washing with water. Washing was then commenced with 0.5N HCl to convert the anion exchanger to Cl^- form. The exchanger was then washed with water followed by 0.5N NaOH till the washings were chloride free. The exchanger was neutralized with 1N CH_3COOH and then stored in a large excess of 0.01M EDTA-NaOH, pH 7.5, which was the usual equilibrating buffer. The buffer was frequently decanted off and fresh buffer added for

complete equilibration.

For packing into chromatographic columns, the gel slurry was poured into the columns and allowed to settle under gravity for 5 minutes. The outlet was then opened to allow a gentle flow. More slurry was added so that a packed column of required length was obtained. The top of the bed was protected with filter paper. The column was then washed with buffer till equilibration.

Preparation of Sephadex cation exchanger, CM-Sephadex, column

Like DEAE-Sephadex, CM-Sephadex was allowed to swell in water and the fines were removed. The gel was washed with 1M and 2M NaCl as before. The exchanger was then successively washed with 0.5N HCl, water, 0.5N NaOH, water, 0.5N HCl and water. The gel was neutralized with 0.5N NaOH and then stored in the equilibrating buffer.

Packing and equilibrating the column was done in the same way as with DEAE-Sephadex column.

Preparation of DEAE- and ECTEOLA Cellulose columns

DEAE- and ECTEOLA Cellulose were regenerated by successive washings with 1N NaOH, water, 1N HCl, water and 1N NaOH (Peterson & Sober, 1956). They were then washed with water to remove excess of NaOH, neutralized with 1N CH_3COOH and were finally equilibrated with the usual 0.01M EDTA-NaOH buffer, pH 7.5.

For packing the chromatographic column, the exchanger suspension was added to the column filled with the equilibrating buffer through a funnel fixed vertically to the column. The total amount of exchanger needed for the column of appropriate length was added to the funnel in one lot. The slurry in the funnel was mechanically stirred. As the exchanger settled slowly, the outlet was opened to give a slow flow rate. After packing, the top of the exchanger bed was protected with a filter paper cut to the proper size. Washing with buffer was continued till equilibration (Sober, Gutter, Wyckoff & Peterson, 1956).

Determination of protein.

Proteins were generally determined by spectrophotometric and refractometric methods.

(a) Spectrophotometric method. For ordinary work and for following proteins in column effluents, absorbance at 280 mμ was measured. For calculating the protein content it was assumed that the absorbance at 280 mμ of a 1% protein solution is 10 per cm light path. This only gives a rough value for protein content since the absorbance of a 1% protein solution can vary between 5 and 19 (Long, 1961).

In some experiments high concentrations of mercaptoethanol (approximately 0.1M) were used. Under these conditions the determination of protein by measuring the absorbance at 280 mμ was difficult because mercaptoethanol formed strongly absorbing compounds, presumably by oxidation, during chromatography. If care was taken to keep the buffer solution containing mercaptoethanol, out of contact with air then this interference could be minimized but not eliminated.

(b) Refractometric method. For the determination of specific activity at the final stages of purification, the protein content was obtained from the refractive index of the protein solution. For the measurement of refractive increment of the protein solution a differential refractometer designed by Cecil & Ogston (1951) was used.

Enzyme solutions were dialysed extensively against the required buffer and readings in the refractometer were taken of buffer against buffer and of enzyme against buffer. From the refractive difference so obtained, the refractive increment of the protein solution was calculated and then the concentration of the protein solution was obtained by assuming a specific refractive increment of protein of 0.001800 $dl.g^{-1}$ at 578 mμ. The ratio of protein content determined spectrophotometrically to that determined refractometrically was 1.67:1.00.

The protein content measured refractometrically is very reliable since the specific refractive increment of simple proteins is constant and has been found to vary only between 0.001842 and 0.001880 $dl.g^{-1}$ (Doty & Edsall, 1951; Doty & Geiduschek, 1953).

The usual chemical methods of protein determination using biuret or Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951) were not used since the biuret method is no more accurate than the spectrophotometric method and the use of Folin-Ciocalteu reagent was not permitted because all the enzyme preparations had mercaptoethanol which is a strong reducing agent and so itself reacts with the

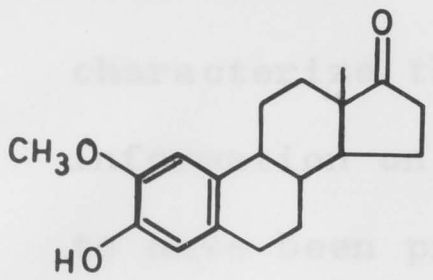
Folin-Ciocalteu reagent.

Sources of Special Materials

2-Methoxyoestrone (I), Oestradiol (II) and Oestriol (III) were gifts from Dr. Roger King of the Imperial Cancer Research Fund, London.

Two samples of adenosine-3',5'-diphosphate (PAP) were used. One was a gift from Dr. E.A. Davidson of the Duke University Medical Center, North Carolina, U.S.A.; and the other from Prof. J. Baddiley of King's College, University of Durham, England.

Equilin (IV), Equilenin (V), 2-Hydroxy-phenanthrene (Phenanthrol, VI) and 15,16 - Dihydro-3-acetoxy-17-oxocyclopenta [a] phenanthrene (acetoxycyclopentenophenanthrene, VII) were gifts from Dr. M.M. Coombs of the Imperial Cancer Research Fund, London. As suggested by Dr. Coombs, acetoxycyclopentenophenanthrene was hydrolyzed to hydroxycyclopentenophenanthrene by refluxing with a 10% KOH solution in 90% ethanol under nitrogen. After 1 hr. the ethanol was removed by heating in an atmosphere of nitrogen. The viscous liquid was diluted with water and then acidified with HCl. The precipitated phenol (hydroxycyclopentenophenanthrene) was centrifuged and



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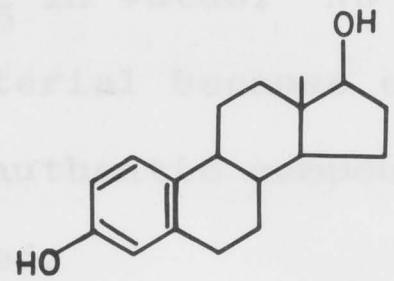

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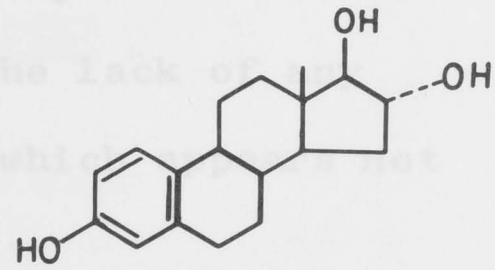

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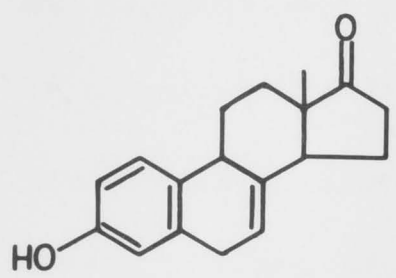

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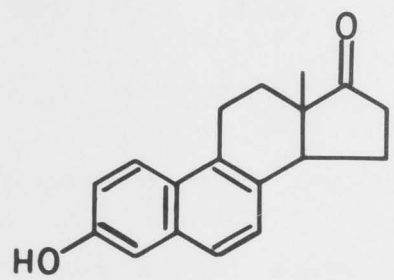

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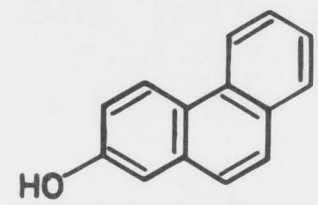

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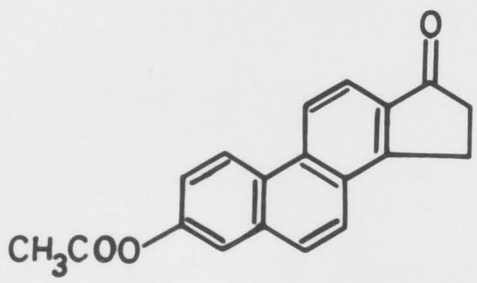


VII

washed 3 times with water to remove Cl^- ions. It was then dried over P_2O_5 in vacuo. No attempt was made to characterize the material because of the lack of any information on the authentic compound which appears not to have been prepared.

CHAPTER 3

ISOLATION OF SULPHOTRANSFERASES

Preparation of Sulphotransferases

Guinea pig liver was used as the source of sulphotransferases. Usually about 150g liver was taken for a preparation. The liver was homogenized in an Omnimixer (Ivan Sorvall Inc., Conn., U.S.A.) with 3 volumes of 0.15M KCl containing 1 mM EDTA (adjusted to pH 7). The homogenate was centrifuged and the supernatant was treated with saturated $(NH_4)_2SO_4$ (60 ml $(NH_4)_2SO_4$ per 100 ml supernatant). The precipitate was rejected and the supernatant was treated with 75 ml saturated $(NH_4)_2SO_4$ per 100 ml of the original supernatant. The precipitate was dissolved in water (0.5 ml per g liver). This gave an enzyme fraction precipitating between the approximate limits of 1.5 and 2.3M $(NH_4)_2SO_4$ (Roy, 1960a). The enzyme solution was then dialysed in the cold for 2 days against several changes of distilled water to destroy the sulphate activating enzymes in the preparation (Robbins, Colowick & Kaplan, 1962). At the end of dialysis the pH of the enzyme solution fell to about 6.2. The pH was adjusted to 5.0 with 0.5N CH_3COOH.

The resulting precipitate was centrifuged off, suspended in 20 ml of 0.03M EDTA-NaOH buffer, pH 7.5, and the pH was readjusted to this figure with 1N Na_2CO_3. After it had stood for 2 hrs in the cold, the mixture was centrifuged and the insoluble material discarded. The supernatant containing the sulphotransferases was made 0.01M in mercaptoethanol and applied to a Sephadex G-200 column (5 x 50 cm) in 0.1M Tris-HCl buffer, pH 7.5, which was developed at room temperature with the same buffer. The sulphotransferases were eluted in a single sharp peak after about 600 ml of buffer had passed through the column. The fraction containing the sulphotransferases were combined and made 0.01M in mercaptoethanol and 0.01M in EDTA by adding the requisite amount of a 5% EDTA solution previously adjusted to pH 7.5 with NaOH. The enzyme solution was then concentrated by ultrafiltration (Sober, Gutter, Wyckoff & Peterson, 1956) to about 5 ml and exhaustively dialysed against a 0.01M EDTA-NaOH buffer, pH 7.5, (0.01 mole disodium EDTA in water adjusted to pH 7.5 with NaOH and made up to 1 litre) containing 0.01M mercaptoethanol. The ionic strength of the buffer is about 0.06 since EDTA exists as a trivalent anion at this pH.

The sulphotransferases were then partially separated by chromatography at 4° on a DEAE-Sephadex A-50 column (1.6 x 30 cm) equilibrated with the above buffer. Elution was carried out with a linear gradient formed from 100 ml of the equilibrating buffer and 100 ml of a similarly prepared buffer (ionic strength, 0.40) containing 0.01M EDTA-0.05M Tris-0.30M sodium acetate containing 0.01M mercaptoethanol. Fractions of 4 ml were collected. Under these conditions, the steroid sulphotransferases, the arylamine sulphotransferase and a small part of the phenol sulphotransferase were eluted between the 4th and the 21st tubes. The further fractionation of these enzymes, referred to as the steroid sulphotransferases is discussed below. After about 100 ml of the gradient had passed through the column, the remaining buffer was removed and the elution was restarted with a buffer of pH 7.5 (ionic strength, 0.52) containing 0.03M EDTA-0.05M Tris-0.30M sodium acetate and 0.01M mercaptoethanol. The major part of the phenol sulphotransferase was eluted around the 12th tube. The complete elution patterns and the further fractionation of the steroid sulphotransferases, discussed below, are presented in Fig. 1.

The two sulphotransferase preparations were

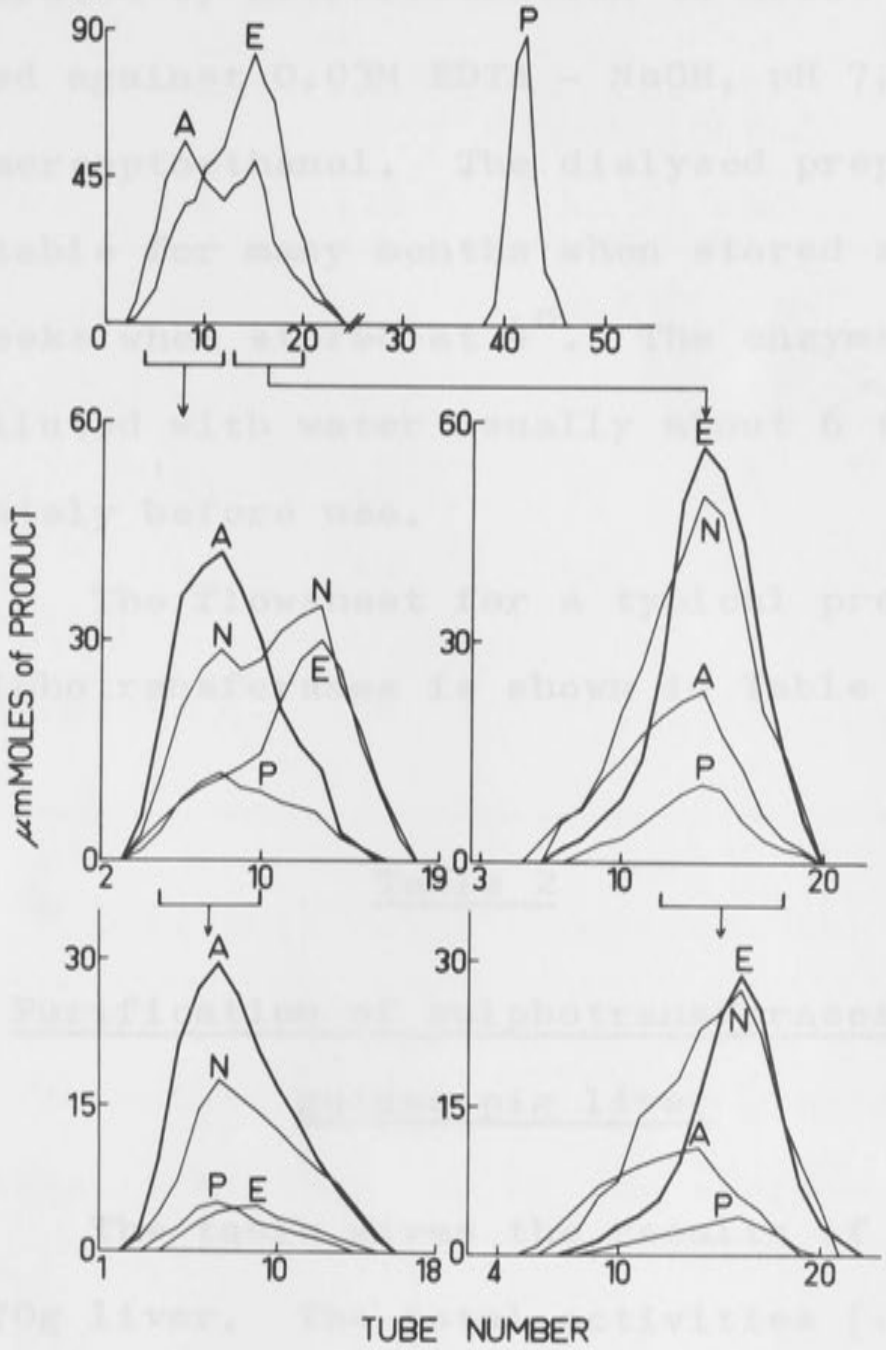


FIG. 1. Separation of sulphotransferase activities (determined as in Table 1) towards androstenolone (A), oestrone (E), p-nitrophenol (P) and 2-naphthylamine (N) on DEAE-Sephadex. Break in abscissa in the top section indicates change of buffer as described in text.

concentrated by ultrafiltration to about 5 ml and dialysed against 0.03M EDTA - NaOH, pH 7.5, containing 0.01M mercaptoethanol. The dialysed preparations were stable for many months when stored at -30^{o} and for many weeks when stored at 4^{o}. The enzyme concentrates were diluted with water usually about 6 times immediately before use.

The flowsheet for a typical preparation of the sulphotransferases is shown in Table 2.

Table 2

Purification of sulphotransferases from guinea pig liver

The table gives the results of a preparation from 170g liver. The total activities (column 1) are expressed as micromoles of sulphate esters formed in 30 mins under the assay conditions in Table 1. The specific activities (columns 2) are expressed as milli-micromoles of sulphate esters formed per $\frac{1}{2}$ hr. per mg of protein. Protein contents were determined from the absorbance at 280 mμ.

	Phenol sulpho-transferase		Androsteno-lone sulpho-transferase		Oestrone sulpho-transferase	
Stage	1	2	1	2	1	2
KCl extract	92	5.1	76	4.2	27	1.5
$(NH_4)_2SO_4$ ppt	57	7.6	69	9.1	27	3.6
pH 5 ppt	53	19	37	14	14	5.0
Sephadex G-200 eluate	42	87	29	62	11	24
DEAE-Sephadex eluate	11*	86	16	85	7.9	105

* Only this value represents the synthesis of p-nitrophenyl sulphate by the phenol sulphotransferase alone, the values at the preceding stages representing the synthesis by this and by the steroid sulphotransferases.

Phenol sulphotransferase

Clearly from Fig. 1 enzymes capable of transferring the sulphuryl group from PAPS to p-nitrophenol were eluted from DEAE-Sephadex in two distinct fractions, a major fraction eluting at the higher ionic strength and referred to as phenol sulphotransferase and a minor fraction associated with the steroid sulphotransferases.

Steroid sulphotransferases

As seen above, the steroid sulphotransferase fraction was eluted from the DEAE-Sephadex column using a concentration gradient of sodium acetate in 0.01M EDTA at pH 7.5. This fraction was capable of sulphurylating p-nitrophenol and 2-naphthylamine. However, even on one passage through DEAE-Sephadex partial separation of the various activities was obtained as shown on the top section of Fig. 1. The androstenolone sulphotransferase peak was eluted ahead of the oestrone sulphotransferase peak. The two peaks were separated, concentrated by ultrafiltration and dialysed against the starting buffer for rechromatography on DEAE-Sephadex, exactly as before, giving the elution pattern shown in the centre of Fig. 1. The androstenolone and oestrone sulphotransferase peaks were separated, concentrated as before and dialysed against the starting buffer. On rechromatography on DEAE-Sephadex a pattern shown in the bottom section of Fig. 1 was obtained. The results clearly showed the separate identity of androstenolone and oestrone sulphotransferases although their complete separation was not achieved.

Fig. 1 also showed that both the androsteno-lone and oestrone sulphotransferases were capable of catalyzing the formation of 2-naphthyl sulphamate and p-nitrophenyl sulphate. This contention is given support by Fig. 2 in which the ratios in the various fractions obtained during the second passage through DEAE-Sephadex (shown in Fig. 1) of the amounts of 2-naphthyl sulphamate produced to the corresponding amounts of, firstly, androstenolone sulphate, secondly, oestrone sulphate, and thirdly, androstenolone plus oestrone sulphate, are plotted. Fig. 3 gives similar ratios for p-nitrophenyl sulphate and the steroid sulphates. Only when the naphthyl sulphamate or the p-nitrophenyl sulphate was compared with the total steroid sulphate - that is, androstenolone sulphate + oestrone sulphates - were constant ratios obtained. Confirmation of this constancy was obtained by considering all the appropriate ratios obtained from the chromatograms shown in Fig. 1 and randomising them into two groups : application of Student's 't' test showed that the two group means were not significantly different even at the 50% level. For 17 degrees of freedom the value of t was 0.42 in the case of the ratios of 2-naphthyl sulphamate to the

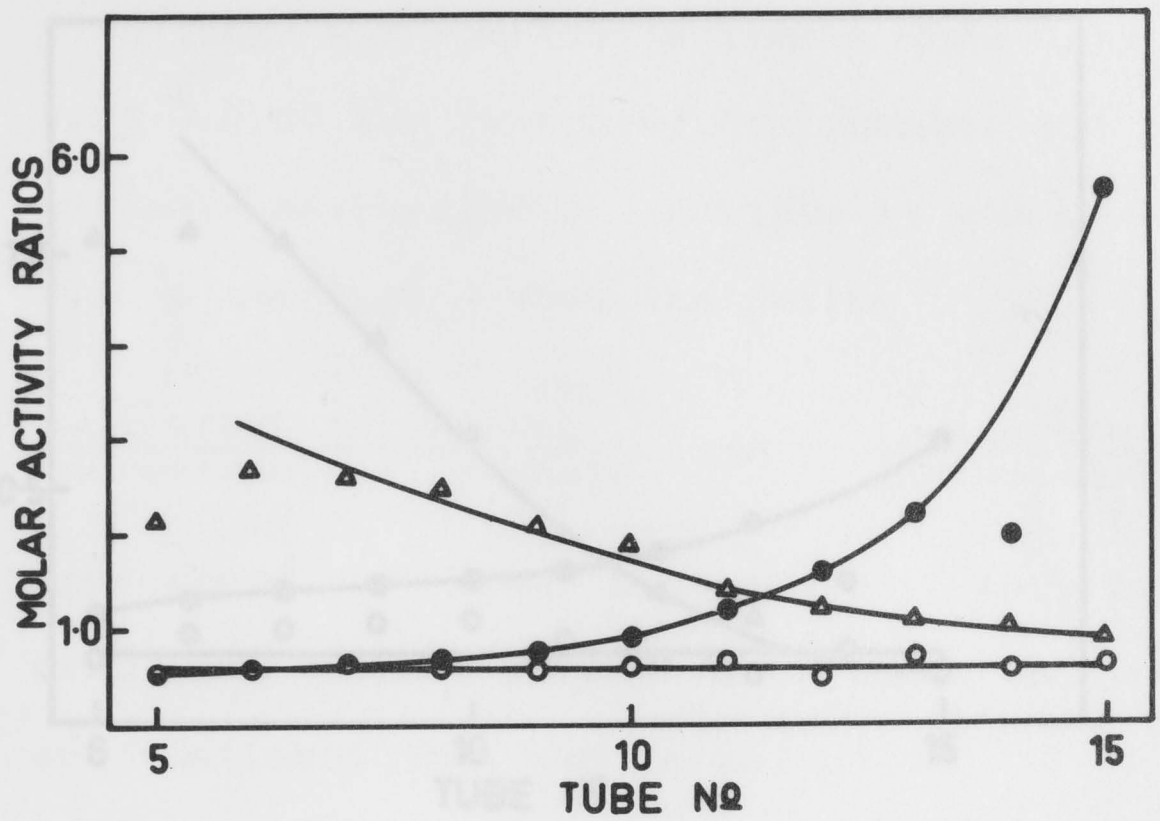


FIG. 2. Amount of 2-naphthyl sulphamate synthesis in each of the appropriate fractions of the centre section of Fig. 1 expressed as a ratio with the amounts of androstenolone sulphate (●), oestrone sulphate (△), and androstenolone + oestrone sulphates (○) similarly synthesized.

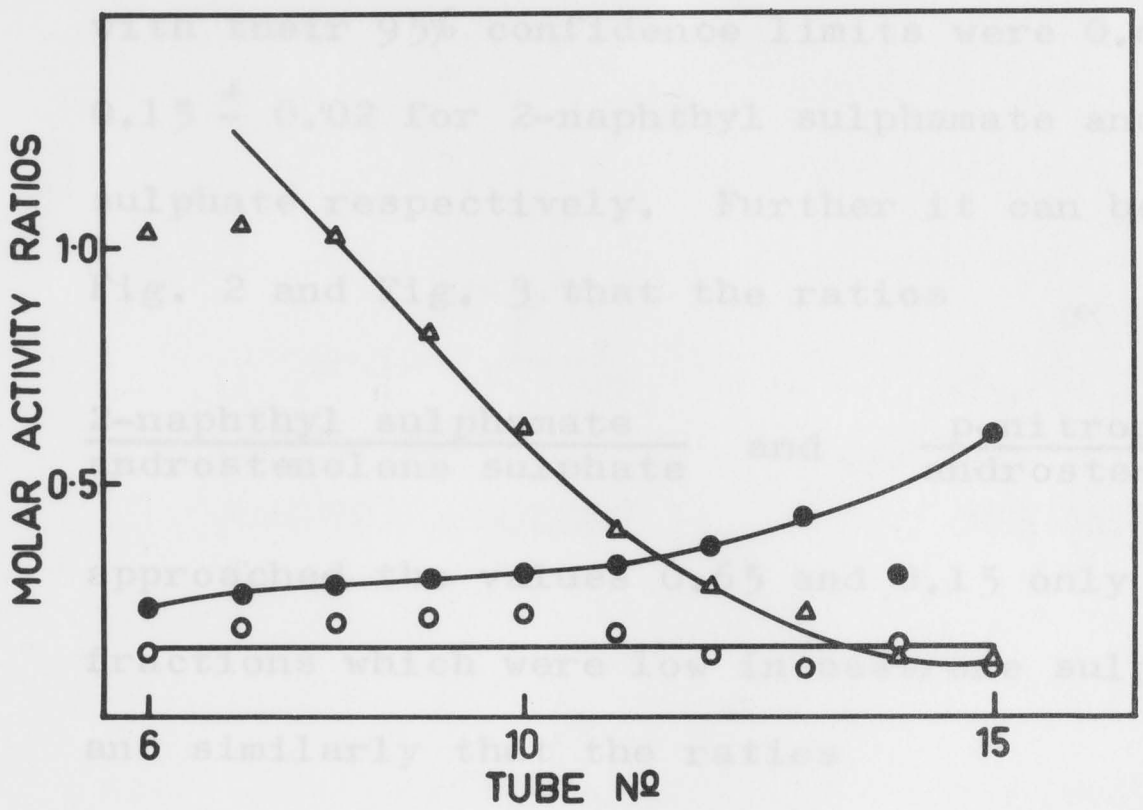


FIG. 3. Amount of p-nitrophenyl sulphate synthesis in Fig. 1 expressed as in Fig. 2.

total steroid sulphate and 0.55 for the corresponding ratios with p-nitrophenyl sulphate. The mean ratios with their 95% confidence limits were 0.65 $\pm$ 0.05 and 0.15 $\pm$ 0.02 for 2-naphthyl sulphamate and p-nitrophenyl sulphate respectively. Further it can be seen from Fig. 2 and Fig. 3 that the ratios

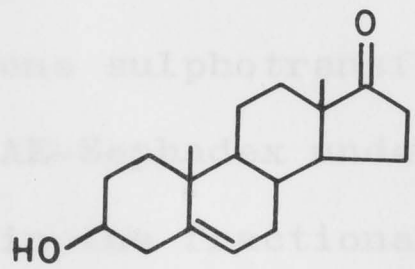
$$\frac{\text{2-naphthyl sulphamate}}{\text{androstenolone sulphate}} \quad \text{and} \quad \frac{\text{p-nitrophenyl sulphate}}{\text{androstenolone sulphate}}$$

approached the values 0.65 and 0.15 only in the initial fractions which were low in oestrone sulphotransferase, and similarly that the ratios

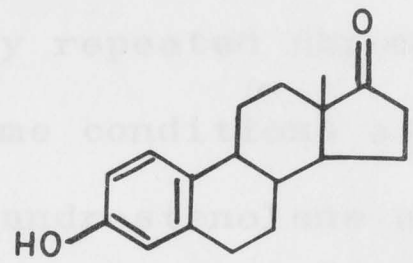
$$\frac{\text{2-naphthyl sulphamate}}{\text{oestrone sulphate}} \quad \text{and} \quad \frac{\text{p-nitrophenyl sulphate}}{\text{oestrone sulphate}}$$

approached the values 0.65 and 0.15 only in the later fractions which were low in androstenolone sulphotransferase. These results strongly suggest that both androstenolone and oestrone sulphotransferases can act as arylamine sulphotransferase and phenol sulphotransferase. If the steroid sulphotransferases do not have this multiple specificity then the various individual enzymes must be very closely, and one might think, rather specifically associated.

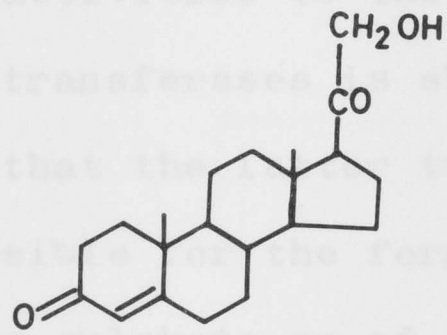
There were two other steroid sulphotransferase activities which were found in both the androstenolone



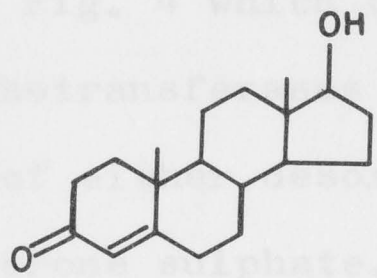
ANDROSTENOLONE



OESTRONE



DESOXYCORTICOSTERONE



TESTOSTERONE

and oestrone sulphotransferase fractions. These were the sulphurylating activities towards desoxycorticosterone and testosterone. Attempts were made to separate these activities from androstenolone and oestrone sulphotransferases by repeated chromatography on DEAE-Sephadex under the same conditions as were used in the fractionation of androstenolone and oestrone sulphotransferases. The relationship of these activities to the androstenolone and oestrone sulphotransferases is shown in Fig. 4 which clearly shows that the latter two sulphotransferases were not responsible for the formation of either desoxycorticosterone sulphate or of testosterone sulphate. Fig. 4 also shows that the rates of synthesis of these two esters closely followed each other maintaining a constant ratio. Table 3 gives the ratio

$$\frac{\text{testosterone activity}}{\text{desoxycorticosterone activity}}$$

at the different stages of purification. Both the "within batch" and "between batch" constancy of this ratio strongly suggest that only a single sulphotransferase is involved in the formation of testosterone and desoxycorticosterone sulphates.

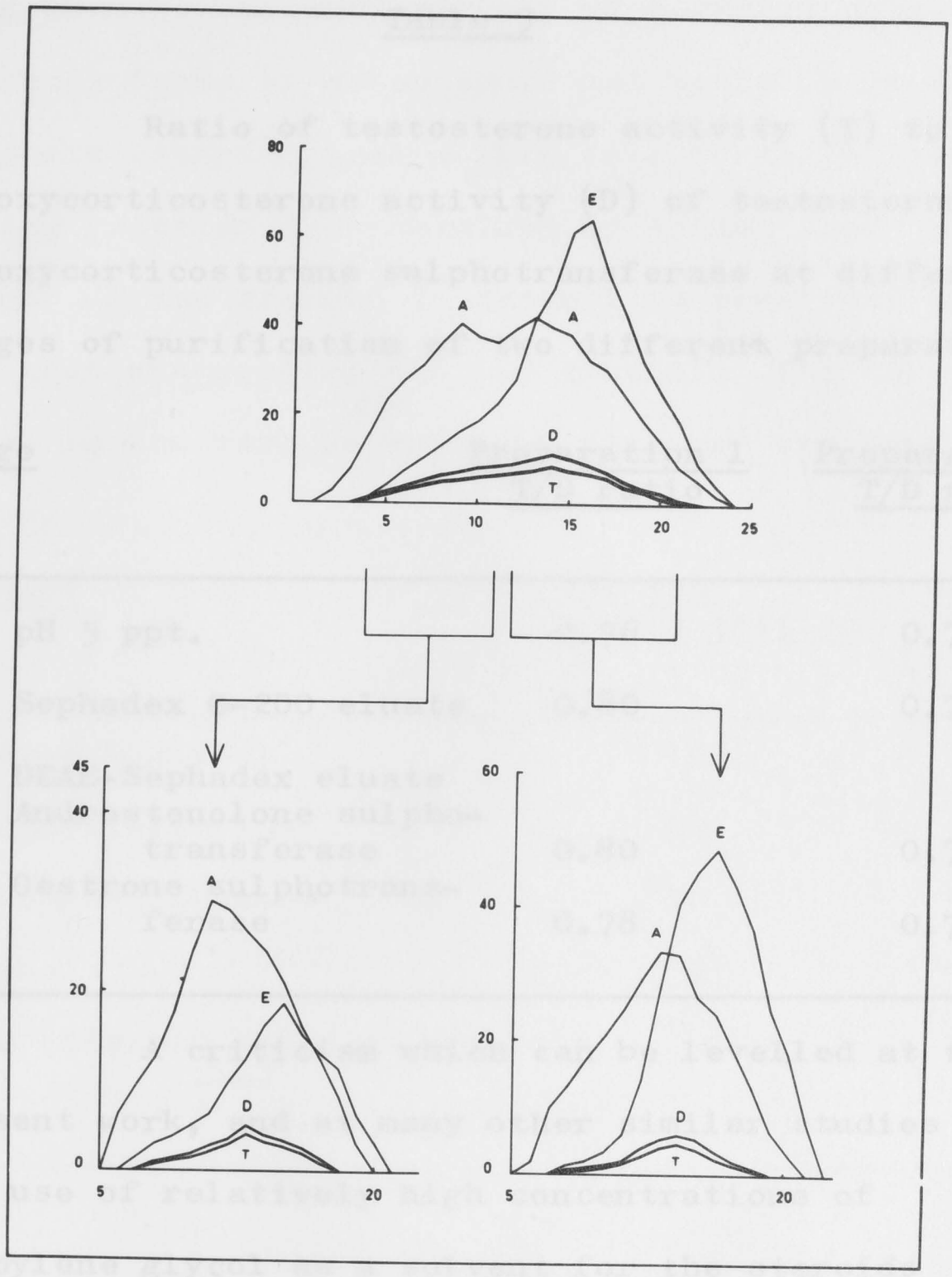


FIG. 4. Separation of sulphotransferase activities towards androstenolone (A), oestrone (E), desoxycorticosterone (D), and testosterone (T) on DEAE-Sephadex using assay conditions of Table 1. Like androstenolone and oestrone; desoxycorticosterone and testosterone were added in propylene glycol. Bottom section of the figure shows results of rechromatography of the indicated fractions after concentration.

Table 3

Ratio of testosterone activity (T) to desoxycorticosterone activity (D) of testosterone/desoxycorticosterone sulphotransferase at different stages of purification of two different preparations.

Stage	Preparation 1 T/D ratio	Preparation 2 T/D ratio
(a) pH 5 ppt.	0.76	0.75
(b) Sephadex G-200 eluate	0.80	0.74
(c) DEAE-Sephadex eluate		
Androstenolone sulphotransferase	0.80	0.77
Oestrone sulphotransferase	0.78	0.73

A criticism which can be levelled at the present work, and at many other similar studies is the use of relatively high concentrations of propylene glycol as a solvent for the steroids because it is known (Spencer, 1960) that this can form a sulphate ester and so can compete for the sulphate donor. That such a criticism is unjustified in the present instance is shown by the fact that the rate of synthesis of androstenolone sulphate was greater when the steroid was added in propylene glycol

than when it was added in any of a number of other solvents (some of which could not possibly form sulphate esters) or as a thin film of solid steroid in the reaction tube obtained by adding the requisite amount of the steroid in ehtanol to the reaction tube and then evaporating off the ethanol. The relative rates in the various reaction mixtures are tabulated below.

Solvent	Solvent concentration % v/v	Relative reaction rate.
Propylene glycol	10	1.00
Dimethylformamide	10	0.99
Dimethylsulphoxide	10	0.96
Ethanol	2	0.82
None (steroid added as thin film)	0	0.84

Discussion.

The methods described above provide a useful means of separating the various sulphotransferases of guinea pig liver : phenol sulphotransferase can be obtained free from any steroid sulphotransferase activity, whereas androstenolone and oestrone

sulphotransferases can be partially separated. During the preliminary work on isolation considerable difficulty was encountered, especially during the later stages of purification, because of the great susceptibility of the enzymes towards metal inactivation. Chromatography on DEAE-Sephadex was invariably accompanied by complete loss of enzymatic activity. The difficulty was however overcome by keeping the enzymes in EDTA and 0.01M mercaptoethanol because in the absence of the latter a slow reversible inactivation took place.

It is clear from Fig. 1 that the synthesis of p-nitrophenyl sulphate is catalyzed by at least three enzymes, the main one being a specific phenol sulphotransferase and the other two being steroid sulphotransferases as discussed below.

With regard to the steroid sulphotransferases the present work confirms the separate identity of androstenolone and oestrone sulphotransferases first noted by Nose & Lipmann (1958). More detailed studies of these enzymes must await their complete separation, a procedure which will probably be complicated by the fact that androstenolone sulphotransferase forms an interacting system. This is suggested

by the data of Fig. 1, which show that the androstenolone sulphotransferase zone gives a consistent trailing whether in the main androstenolone sulphotransferase fraction where it trails backward or in the oestrone sulphotransferase fraction where it trails forward. As it will be shown and discussed in a later chapter, the situation involves an interaction, possibly through disulphide bonds, between androstenolone and oestrone sulphotransferases.

As pointed out already, it seems to have been shown beyond doubt that androstenolone and oestrone sulphotransferases can both act as phenol sulphotransferase and arylamine sulphotransferase forming p-nitrophenyl sulphate and 2-naphthyl sulphamate respectively. Definite proof of this hypothesis will be hard to obtain; but it is difficult to see what other interpretation can be put on the data. Certainly if the two sulphotransferases do not both show these other type of sulphotransferase activity then the several enzymes must be very closely associated.

The present study therefore suggests that the sulphotransferases of liver may not be as numerous as has been thought. In guinea pig there certainly exist a phenol sulphotransferase, an androstenolone

sulphotransferase, an oestrone sulphotransferase and a desoxycorticosterone sulphotransferase, the last named perhaps also acting as testosterone sulphotransferase. Further, both androstenolone and oestrone sulphotransferases seem to accept p-nitrophenol and 2-naphthylamine as substrates. It is therefore extremely probable that the postulated arylamine sulphotransferase does not have a separate existence - its activity is only reproduced by androstenolone and oestrone sulphotransferases.

CHAPTER 4

SOME PROPERTIES OF SULPHOTRANSFERASES

Introduction

It was seen earlier that by chromatography on DEAE-Sephadex, sulphotransferases of guinea pig liver were separated into a specific phenol sulphotransferase and a group of enzymes which were called the steroid sulphotransferases. The steroid sulphotransferases were also partially separated into two major fractions, namely androstenolone and oestrone sulphotransferases both of which could presumably also act as arylamine and phenol sulphotransferases. For further purification of all these enzyme fractions it was necessary to know some of the properties of the enzymes. Also it is well known that several different enzymes capable of degrading PAPS (PAPS-degrading enzymes) are widely distributed. The presence of such enzymes in the sulphotransferase preparations would greatly complicate the assays of the latter and hence it was important to investigate the possible occurrence of the degradative enzymes in the various liver fractions.

Experimental

As already pointed out sulphotransferases are rather susceptible to metal inhibition. It was therefore necessary in many cases to use dialysis tubing which had been treated to remove possible metal contamination. This was carried out as follows. Visking dialysis tubings were first cleaned by heating them in distilled water on a steam bath for 1 hr. The heating was repeated with two more changes of water. The tubes were then taken out and washed with glass distilled water and soaked in several changes of glass distilled water over a period of 24 hrs (Hughes & Klotz, 1956). The cleaned dialysis tubes were then rendered metal free by placing them in a 3% solution of redistilled acetic acid in glass distilled water, heated to 60^{o}, which was then allowed to cool to room temperature. The dialysis tubings were then washed several times with glass distilled water to remove all acid and were finally stored in glass distilled water.

These dialysis tubings were used to study the effect of the removal of EDTA and mercaptoethanol from the enzyme. Obviously for these experiments all buffers not containing EDTA were made up with glass distilled water and all glassware was freed from metal

ions by three rinsings with 3% acetic acid followed by several washings with glass distilled water.

PAPS-degrading Enzymes : The detection of PAPS degrading enzymes in phenol sulphotransferase was carried out as follows. A known amount of PAPS was preincubated with the enzyme for 5 min at 37° in the usual reaction mixture from which p-nitrophenol had been omitted. At the end of this time, p-nitrophenol was added. Progress curves for the formation of p-nitrophenyl sulphate were obtained using the PAPS pretreated as described above and also using untreated PAPS which served as control.

In another series of experiment a known amount of PAPS was preincubated with the enzyme for 1 hr. at 37°. The transferrable sulphate was then determined. The transferrable sulphate of unincubated PAPS served as control. The difference in the two readings gave the amount of transferrable sulphate lost during preincubation.

Samples of phenol sulphotransferase and steroid sulphotransferases were sent to Dr. J.G. Jones of the Biochemistry Department of the University College of South Wales, Cardiff, who had kindly agreed to test the samples for PAPS-degrading enzymes. Known

amounts of [^{35}S] PAPS were incubated with the above enzymes (in about double the concentration used with the normal sulphotransferase assay) for 50 min. At the end of incubation the reaction tubes were immersed in boiling water for 2 min and then 5 microlitre samples were examined by horizontal paper electrophoresis (with 0.1M acetate buffer at pH 4.5 and a potential gradient of 10 volts/cm) for 2 hrs. The dried electrophoretograms were then scanned in an Actigraph Automatic Chromatogram Scanner (Nuclear Chicago) to detect [^{35}S] PAPS, [^{35}S] APS and $^{35}SO_4^{2-}$ ions.

Effect of Vitamin A on Enzyme Activity : Solutions of retinol (vitamin A) and retinyl acetate (vitamin A acetate) were made up in ethanol (1 mg/25 ml). 0.02 ml of the solutions was added to the various assays of phenol and steroid sulphotransferases. A control assay was carried out in which 0.02 ml ethanol was added to the various reaction mixtures to include the effect of ethanol. The results from the various assays then directly gave the effect of vitamin A on the sulphotransferases.

Acetone and $(NH_4)_2SO_4$ Fractionations : The experimental methods for fractionations by acetone and $(NH_4)_2SO_4$

have been described earlier.

Electrophoresis on Starch Gel : Electrophoresis of phenol sulphotransferase on starch gel was very kindly conducted by Mrs. E. Allen. The electrophoresis was carried out for 6 hrs at 180 volts in 0.014M Tris-citric acid buffer, pH 7.5. At the end of electrophoresis the slab was sliced longitudinally and one half was dyed with 0.1% nigrosine. By comparison with the bands in the stained slab, the test slab was cut into fractions. These were assayed for sulphotransferase activity by adding them to the usual reaction mixture for the determination of phenol sulphotransferase. It seemed impossible to devise a method whereby its activity could be detected on the gel itself.

Isoelectric Fractionation : The apparatus obtained from LKB-Produkter, Stockholm, consists of an electrolysis column of 110 ml capacity with a cooling jacket and two platinum electrodes. The method consists in setting up a sucrose density gradient column in the presence of carrier ampholytes which are a mixture of many synthetic low molecular weight aliphatic poly-amino-poly-carboxylic acids. When such a complex mixture of isomers and homologues is

subjected to electrolysis an essentially linear pH gradient, stabilized by the density gradient, is set up because the component ampholytes display a whole spectrum of pK and so of pI (isoelectric point) values (Vesterberg & Svensson, 1966). Now if a protein is added somewhere in such a column and electrolysis is started, then, as a stable pH gradient is established by electrolysis, the protein will migrate either towards the anode or towards the cathode, depending on its pI, and finally remain steady at its pI. The protein is then said to be focussed at its isoelectric point. When the steady state is achieved there is an equilibrium between diffusion and electric migration for the proteins as well as for the carrier ampholytes (Svensson, 1961) and such a steady state is characterized by a stable pH gradient with focussed isoelectric protein zones.

For carrying out isoelectric fractionation the enzyme was dialysed against 0.5% ampholyte 5-8, pH 6.8, containing 0.01M mercaptoethanol. The enzyme activity was unchanged after such dialysis for two days.

A sucrose density gradient was then set up in the column. The procedure adopted was exactly that of

Vesterberg & Svensson (1966). A dense solution was prepared by dissolving 7.5 ml of ampholyte 5-8 in 60 ml of a stock solution containing 500g sucrose per litre. The less dense solution was made up by taking 2.5 ml of the ampholyte and adjusting the volume to 60ml with distilled water. Both the dense and light solutions were made 0.01M in mercaptoethanol. These two solutions were used to set up the required density gradient in the electrolytic apparatus at 4^{o} exactly as described by Vesterberg & Svensson (1966). The ampholytes were protected from anodic oxidation and cathodic reduction by the addition of phosphoric acid and ethylene diamine to the anode and cathode respectively. Electrolysis was carried out at an energy input of 1 watt : to maintain this the voltage had to be increased from time to time till a steady state was reached (about 2 days) when the voltage stabilized at 1000 volts and the current at 1 mA.

At the end of electrolysis, 2 ml fractions were collected for assays. The pH of the fractions were measured.

Molecular Weight by Gel Filtration : A column (1.5 x 48 cm) of Sephadex G-200 was set up and equilibrated with a veronal buffer, pH 7.5 (prepared by dissolving

4.12g sodium barbitone, 7.36g barbitone and 10.53g sodium chloride in hot water and, after cooling, making up to 2 litres with water). The enzyme and some pure proteins of known molecular weights were eluted from the column (Andrews, 1964) using the same buffer. 2 ml fractions were collected by hand and the elution volumes of the enzyme peak (by assay) and the protein peaks (by absorbance at 280 mμ) for the others were noted. The molecular weight of the enzyme was then obtained by comparison.

RESULTS

1. General

PAPS-degrading Enzymes : The progress curves obtained by using normal PAPS (Curve 1) and PAPS preincubated with phenol sulphotransferase (Curve 2) are presented in Fig. 5. The two curves are essentially parallel showing that no significant breakdown of PAPS occurred during preincubation.

The results of transferrable sulphate

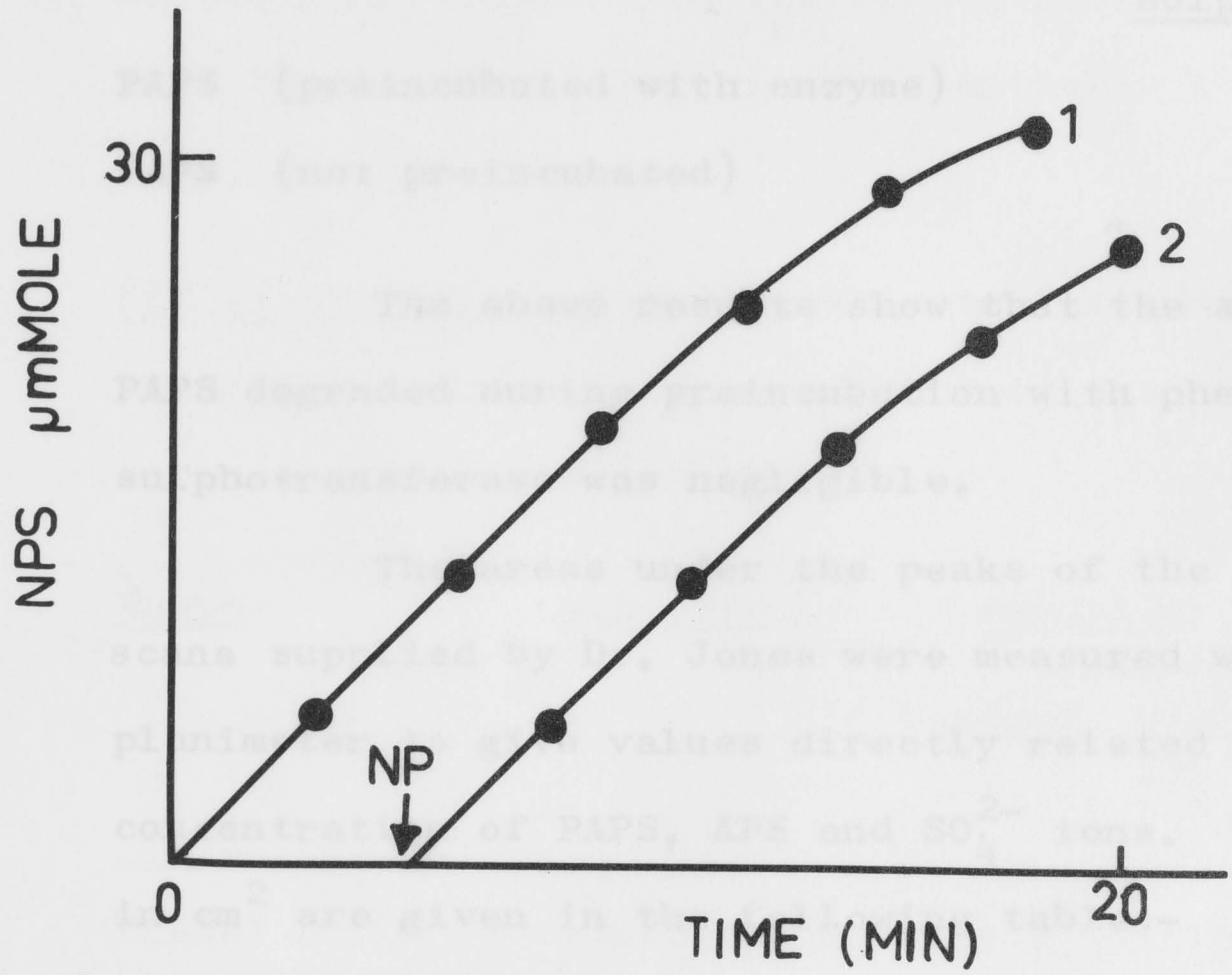


FIG. 5. Progress curves using (1) normal PAPS and (2) PAPS preincubated with phenol sulphotransferase.

determination are presented below:-

Conditions	Transferrable sulphate, %
PAPS (preincubated with enzyme)	78
PAPS (not preincubated)	80

The above results show that the amount of PAPS degraded during preincubation with phenol sulphotransferase was negligible.

The areas under the peaks of the Actigraph scans supplied by Dr. Jones were measured with a planimeter to give values directly related to the concentration of PAPS, APS and SO_4^{2-} ions. The areas in cm^2 are given in the following table:-

	PAPS	APS	SO_4^{2-}
Control	10.9	0.6	0.4
Androstenolone sulphotransferase	2.4	6.2	4.1
Oestrone sulphotransferase	7.0	1.5	3.9
Phenol sulphotransferase	10.5	0.8	0.8

It is clear once again that phenol sulphotransferase contains no significant PAPS-degrading activity but that these degradative enzymes are present to significant extents in androstenolone and oestrone sulphotransfer-

ases. The degradation of PAPS obviously follow different routes in the androstenolone and oestrone sulphotransferases : in the former gives APS and SO_4^{2-} ions but in the latter gives essentially only SO_4^{2-} ions.

Effect of Vitamin A : The effects of vitamin A and vitamin A acetate on the activity of the sulphotransferases are tabulated below:-

Enzyme	Acceptor	Amount of sulphate ester formed		
		Control	With retinol	With retinyl acetate
Phenol sulphotransferase	p-Nitrophenol	19.1	19.1	19.1
Androstenolone sulphotransferase	Androstenolone	25.4	25.2	25.5
Oestrone sulphotransferase	Oestrone	31.3	31.1	31.0

The above results show that retinol or retinyl acetate had no effect on the sulphotransferase activities.

Acetone Fractionation : There was a severe loss of enzyme activity during acetone precipitation and the recovery of the enzyme after such treatment ranged from 10 to 20%. What activity was recovered precipitated between 0-16% acetone $^{v}/_{v}$ at -5^{o}.

$(NH_4)_2SO_4$ Fractionation : The results of precipitation with $(NH_4)_2SO_4$ are presented below where A, E and P represent androstenolone, oestrone and phenol sulphotransferases respectively.:-

$(NH_4)_2SO_4$ Saturation	Recovery as % of control		
	A	E	P
0 - 0.3	30	20	25
0 - 0.4	59	70	50
0 - 0.5	87	88	71

It is thus seen that no separation of the sulphotransferases was achieved by $(NH_4)_2SO_4$ fractionation.

2. Phenol Sulphotransferase

As seen earlier enzymes capable of transferring the sulphuryl group from PAPS to p-nitrophenol are eluted from DEAE-Sephadex in two fractions. The

major fraction which is eluted at higher ionic strengths is specific for phenols and is called phenol sulphotransferase whereas the other fraction is associated with the steroid sulphotransferases and is referred to as steroid sulphotransferases. Both fractions formed p-nitrophenyl sulphate at a rate directly proportional to the enzyme concentration but showed zero order kinetics for only a limited time, 10 min and 30 min for the phenol sulphotransferase and steroid sulphotransferase respectively. For kinetic studies to compare the properties of the two fractions a uniform incubation time of 10 min was therefore used.

Effect of Mg^{2+} Ions : A striking difference between the two fractions was their different requirements for Mg^{2+} ions. Apparently the phenol sulphotransferase did not require Mg^{2+} ions for maximal activity whereas the steroid sulphotransferases did. This is clearly shown by the results tabulated below which give the relative rates of formation of p-nitrophenyl sulphate by the two fractions in the presence and absence of Mg^{2+} ions.:-

	Phenol sulphotransferase	Steroid sulphotransferase
With 2 mM excess Mg^{2+} (Table 1)	100	100
Without added Mg^{2+}	101	60

pH Optima : The two fractions also differed in their pH optima (Fig. 6). Phenol sulphotransferase had a pH optimum of 5.8 and steroid sulphotransferase had pH optimum between 7.0 and 7.5. Both the fractions showed optimum activity at about 1 mM p-nitrophenol and had not reached an optimum by 0.2 mM PAPS. (The phenol sulphotransferase was purified further and the detailed kinetic results appear in later chapters).

Stability at Various pH : Equal amounts of phenol sulphotransferase were treated at 37° for 10 min with dilute EDTA buffers of various pH and at the end of this period the enzymes were assayed at pH 5.6 by adding to them the standard reaction mixture for the assay of phenol sulphotransferase from which the enzyme had been omitted.

The activity, expressed as a percentage of control activity, of the enzyme samples pretreated at various pH is tabulated below.:-

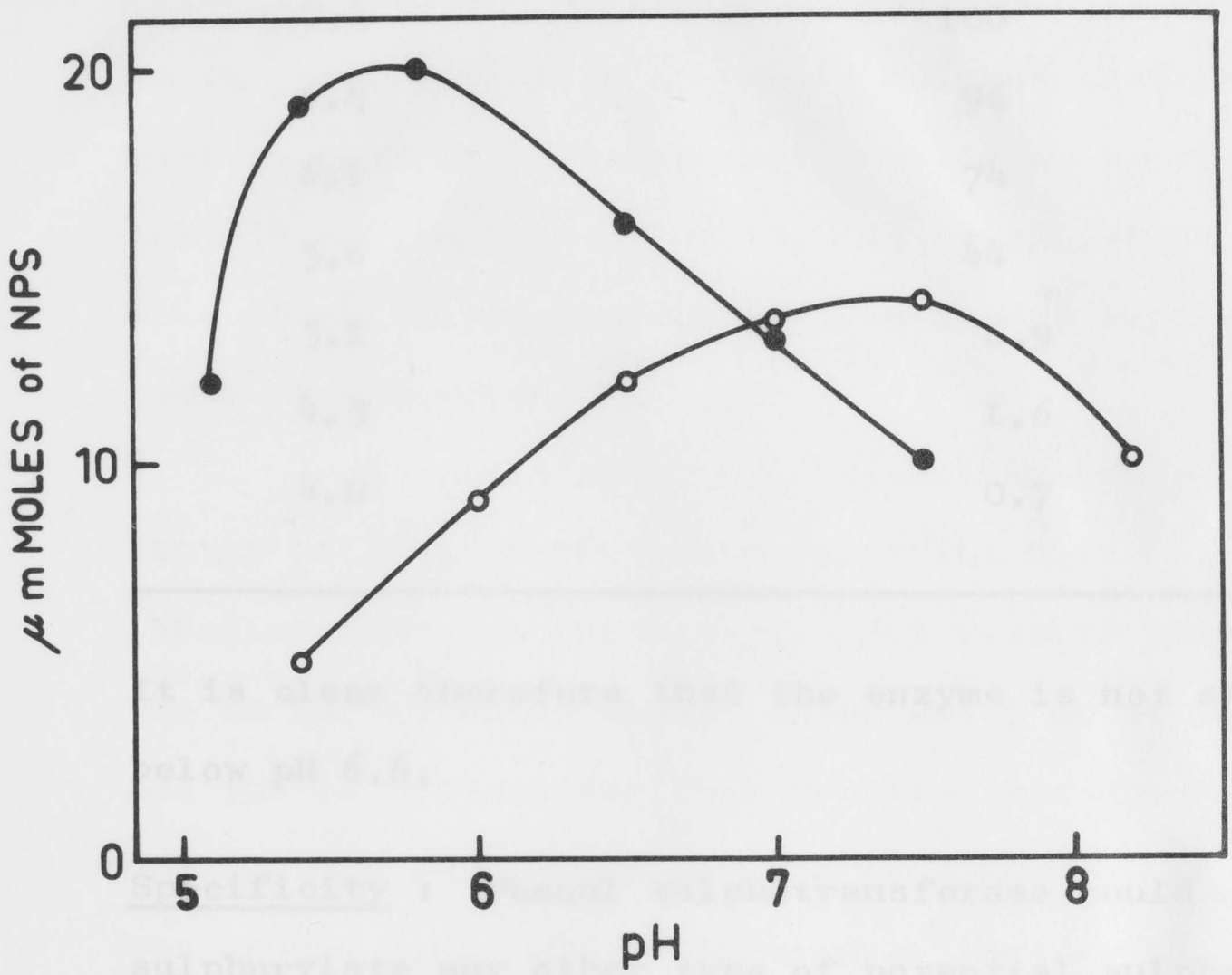


FIG. 6. Effect of pH on the synthesis of p-nitrophenyl sulphate (NPS) by phenol sulphotransferase (●) and unfractionated steroid sulphotransferases (○).

Enzyme at pH	Activity as % control
7.4	100
6.6	96
6.1	74
5.6	44
5.1	6.9
4.5	1.6
4.0	0.7

It is clear therefore that the enzyme is not stable below pH 6.6.

Specificity : Phenol sulphotransferase could not sulphurylate any other type of potential sulphate acceptor tested. A preparation capable of catalyzing the formation of 44 millimicromoles of p-nitrophenyl sulphate in 10 min formed no detectable amounts (less than 0.2 millimicromole) of androstenolone sulphate, oestrone sulphate, testosterone sulphate, desoxyoestrone sulphate and 2-naphthyl sulphamate even on prolonged incubation.

With regard to tyrosine as substrate, the methylene blue method could not be used since the method does not detect tyrosine sulphate which is too polar to

give a chloroform soluble methylene blue salt.

However, tyrosine methyl ester was found to act as a competitive inhibitor of phenol sulphotransferase with respect to p-nitrophenol showing an apparent inhibition constant of 20 mM. It is therefore possible that phenol sulphotransferase can accept tyrosine methyl ester as substrate.

A sample of phenol sulphotransferase was tested by Jones (1966) in Cardiff who used ^{35}S labelled PAPS for the assays. His results show that the Km for tyrosine methyl ester was 5 mM at pH 7.5 and the pH optimum for this reaction was about 8.6 whereas the pH optimum for the transfer to p-nitrophenol was around 6.0. The differencesin the two pH optima are explained by the fact that tyrosine derivatives can only be sulphurylated when the amino group is unprotonated, that is at alkaline pH (Segal & Mologne, 1959). On extending the work to the steroid sulphotransferases sent to him, Jones (1966) found that these enzymes could also catalyze the sulphurylation of tyrosine methyl ester possibly through the participation of the associated phenol sulphotransferase activity. With regard to the specificity of phenol sulphotransferase towards various other phenols, detailed studies have been made and the results are presented in a later

chapter.

Removal of EDTA and Mercaptoethanol : One aliquot of the enzyme in 0.03M EDTA-NaOH, pH 7.5, containing 0.01M mercaptoethanol was dialysed exhaustively against 0.01M Tris-acetic acid, pH 7.5, containing 0.01M mercapto-ethanol under strictly metal free conditions to remove all EDTA. Another aliquot of the enzyme was dialysed exhaustively against 0.01M Tris-acetic acid, pH 7.5, under metal free conditions to remove both EDTA and mercaptoethanol. The activities were then determined. It was found that the removal of EDTA had no effect on the enzyme activity. The removal of mercaptoethanol resulted in about 35% loss of activity which was completely restored by allowing the enzyme to stand in 0.01M mercaptoethanol for 10 min. The results are tabulated below. The undialysed enzyme served as control. Activities are expressed as millimicromoles of p-nitrophenyl sulphate formed in 10 min.

Enzyme	Activity
1. Control	17.3
2. 0.01M mercaptoethanol - no EDTA	17.1
3. No mercaptoethanol - no EDTA	11.2
4. 3 stood in 0.01 mercaptoethanol for 10 min.	17.4

Electrophoresis on Starch Gel : After electrophoresis of phenol sulphotransferase several protein bands were formed. These bands were cut out as described above and assayed. None of the bands were found to be active.

Determination of Isoelectric Point : By the isoelectric fractionation procedure the enzyme was completely inactivated. A prominent band of precipitated proteins was focussed between pH 5.0 and 5.6 but all the fractions were inactive. Since the enzyme is stable above pH 6.6 all that can be said from these experiments is that the enzyme probably has an isoelectric point below 6.6.

Determination of Molecular Weight : The elution volumes of the enzyme and other proteins are presented in Table 4 below.

Table 4

Elution volumes (V_e) in ml for various proteins.

Protein	Mol. wt.	V_e
Sulphatase A (monomer)	107,000	38
Bovine serum albumin	67,000	48
Ovalbumin	45,000	56
Chymotrypsinogen	25,000	64
Phenol sulphotransferase	67,000(Estimated)	48

The fact that bovine serum albumin and phenol sulphotransferase have identical elution volumes strongly suggest that the latter probably has a molecular weight around 67,000. This value cannot be taken as absolute because in deriving the value it is presupposed that bovine serum albumin and phenol sulphotransferase have similar molecular shapes, gel filtration characteristics being not only sensitive to molecular size but also to molecular shape, although to a smaller extent.

3. Steroid Sulphotransferases

Effect of Mg^{2+} Ions : All the activities shown by the steroid sulphotransferases required the presence of 2 mM Mg^{2+} ions (in excess of the EDTA added with the enzyme to the reaction mixture) for activity. Increasing the Mg^{2+} ion concentration to 10 mM gave no further rise in activity.

pH Optima and K_m Values : The pH-activity curves for the several activities are shown in Fig. 7 and 8 and clearly support the hypothesis that p-nitrophenyl sulphate and 2-naphthyl sulphamate are formed by both androstenolone and oestrone sulphotransferases.

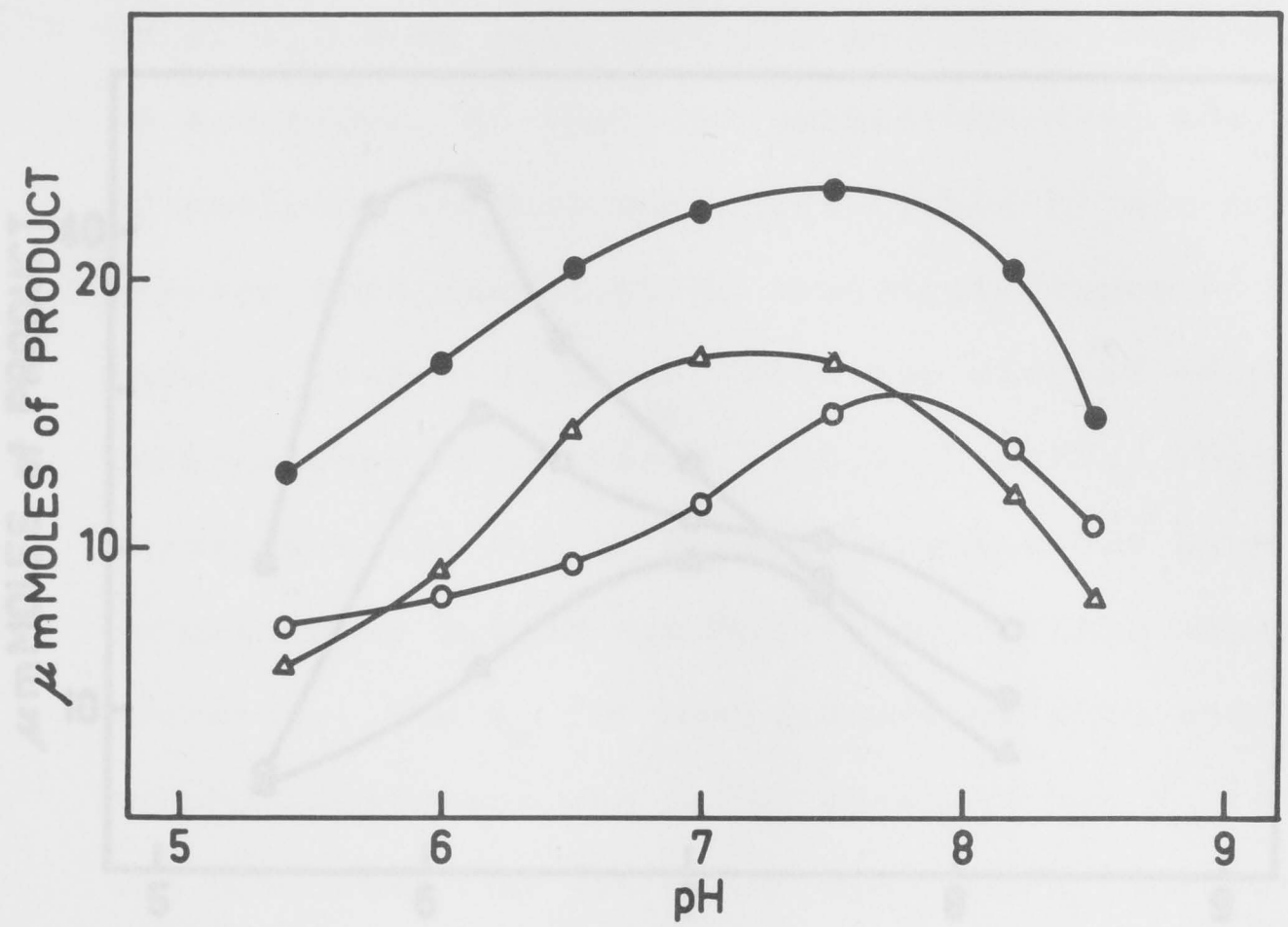


FIG. 7. Effect of pH on the synthesis of androstenolone sulphate (●), 2-naphthyl sulphamate (○) and p-nitrophenyl sulphate (△) by androstenolone sulphotransferase.

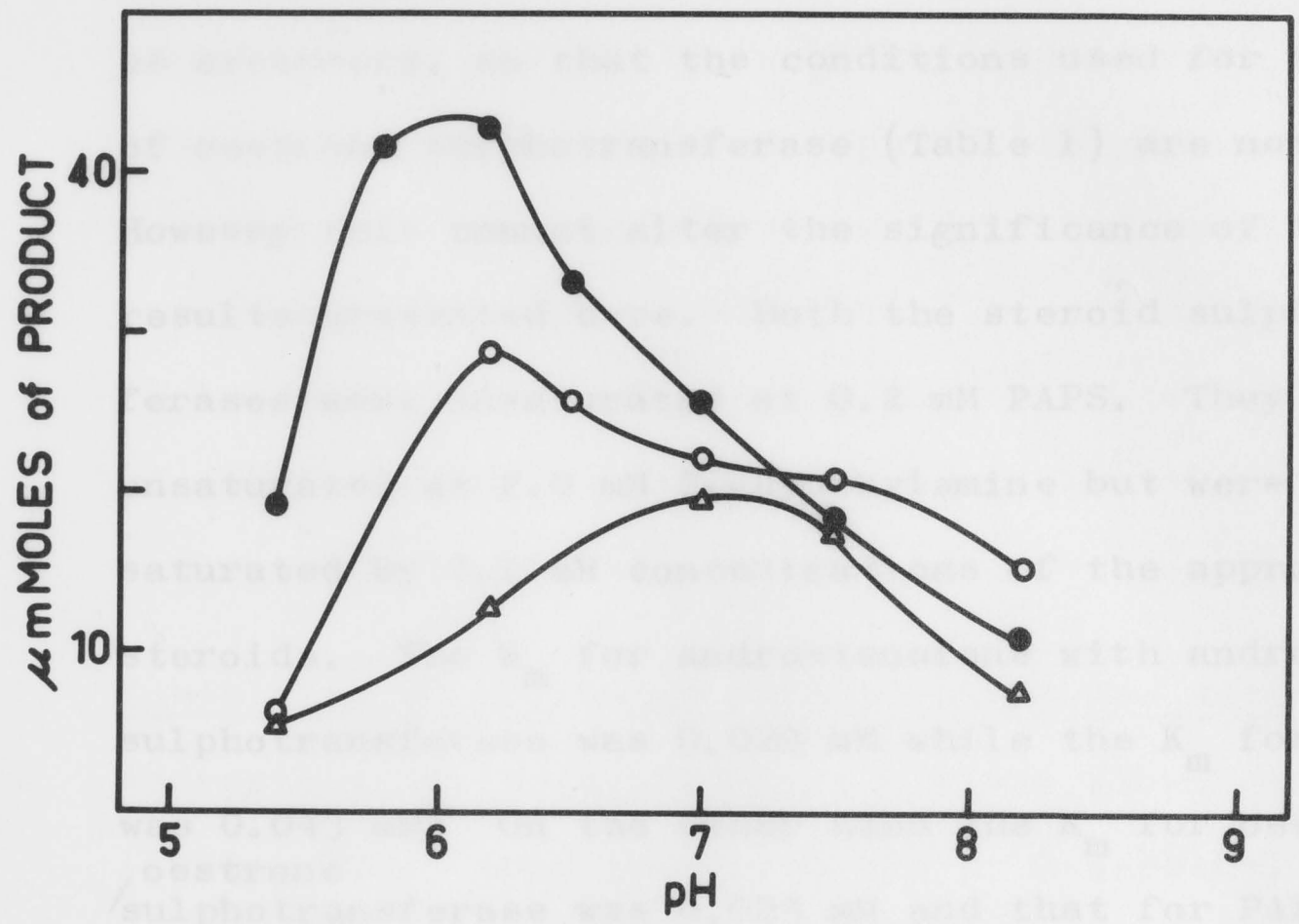


FIG. 8. Effect of pH on the synthesis of oestrone sulphate (●), 2-naphthyl sulphamate (○) and p-nitrophenyl sulphate (△) by oestrone sulphotransferase.

The oestrone sulphotransferase had an optimum pH of 6.2 both with oestrone and with 2-naphthylamine as acceptors, so that the conditions used for the assay of oestrone sulphotransferase (Table 1) are not optimal. However this cannot alter the significance of the results presented here. Both the steroid sulphotransferases were unsaturated at 0.2 mM PAPS. They were also unsaturated at 2.0 mM 2-naphthylamine but were saturated by 0.1 mM concentrations of the appropriate steroids. The K_m for androstenolone with androstenolone sulphotransferase was 0.020 mM while the K_m for PAPS was 0.043 mM. On the other hand the K_m for oestrone with oestrone sulphotransferase was 0.025 mM and that for PAPS was higher than 0.100 mM. This higher K_m value for PAPS was probably caused by the presence of an appreciable quantity of PAPS-degrading enzymes present in the preparations as seen above.

With regard to 2-naphthylamine as substrate there appeared to be a critical substrate concentration of about 0.1mM below which no enzyme activity could be detected under the normal conditions of assay. The usual reciprocal plots of $\frac{1}{v}$ (reciprocal initial velocity) against $\frac{1}{s}$ (reciprocal substrate concentration) were therefore nonlinear, but when $\frac{1}{v}$ was plotted against

$\frac{1}{s-x}$ where x is the critical substrate concentration, straight lines were obtained. These results are therefore similar to those of Roy (1961) who first studied the synthesis of 2-naphthyl sulphamate by guinea pig liver preparations. By using an altered experimental procedure Roy (1961) was also able to show that the reaction did proceed at low concentrations of 2-naphthylamine but that in this region the v against s curve was sigmoidal.

Inhibition of Arylamine Sulphotransferase Activity by Androstenolone Methyl Ether : It was shown by Roy (1960a, 1961) that the synthesis of 2-naphthyl sulphamate by crude preparations from guinea pig liver was powerfully inhibited by small concentrations of 3 -methoxyandrost-5 en-17-one (androstenolone methyl ether) which could not itself be sulphurylated due to the blocking of the hydroxyl group by methylation. The effect of this compound on the more purified enzyme preparations described here was therefore investigated. The results are summarized in Table 5 which shows that 2 μM concentrations of androstenolone methyl ether powerfully inhibited the formation of 2-naphthyl sulphamate by both androstenolone and oestrone sulphotransferases. The compound

also inhibited the synthesis of p-nitrophenyl sulphate by oestrone sulphotransferase but to a smaller extent.

Table 5

The effect of 2 μM androstenolone methyl ether on sulphotransferase activities

The activities were determined as in Table 1, and are expressed as rates relative to that (1.00) found in the absence of the added steroid, the absolute values of which were 27.1 μm moles of androstenolone sulphate and 64.9 μm moles of oestrone sulphate respectively per 30 min per mg protein.

Acceptor substrate	Relative Activity: Androstenolone sulphotransferase	Relative Activity: Oestrone sulphotransferase
Androstenolone	1.00	0.98
Oestrone	0.99	0.99
p-Nitrophenol	1.00	0.75
2-Naphthylamine	0.45	0.49

Specificity : A detailed study of the specificities of the steroid sulphotransferases would clearly be premature at this stage because of their incomplete separation. However, androsterone (VIII) and cholesterol (IX) seem to be substrates for androstenolone sulphotransferase, as shown by the constancy of the ratios of the various activities through the stages of purification (Table 6).

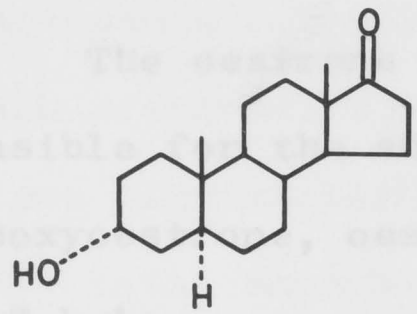
Table 6

Relative steroid sulphotransferase activities with various substrates

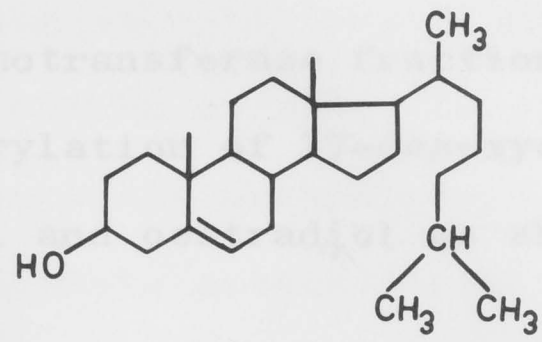
The activities were determined as in Table 1, and the rates of sulphate ester formation with the various steroids are expressed relative to the rates with androstenolone and with oestrone.

		Androsterone	Cholesterol
1. Ratios with androstenolone			
pH 5 ppt.		0.69	0.19
Sephadex G-200 eluate		0.70	0.15
DEAE-Sephadex eluate	a	0.71	0.13
	b	0.79	0.14
2. Ratios with oestrone			
pH 5 ppt.		2.3	0.97
Sephadex G-200 eluate		0.88	0.19
DEAE-Sephadex eluate	a	1.2	0.22
	b	0.55	0.10

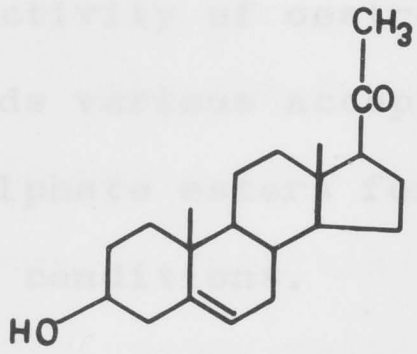
a Androstenolone sulphotransferase fraction
b Oestrone sulphotransferase fraction



VIII



IX



X

Pregnenolone (X) was sulphurylated at the same rate as androstenolone by the androstenolone sulphotransferase fraction.

The oestrone sulphotransferase fraction was responsible for the sulphurylation of 17-desoxyoestrone, 2-methoxyoestrone, oestriol and oestradiol as shown in Table 7 below.

Table 7

The activity of oestrone sulphotransferase fraction towards various acceptors, expressed as millimicromoles of sulphate esters formed per 30 min under the same assay conditions.

Acceptor	Activity
Oestrone	17
17-Desoxyoestrone	10
2-Methoxyoestrone	6.9
Oestradiol	8.2
Oestriol	1.5

From the above preliminary results it appears that oestradiol and oestriol are much poorer substrates

than the ketonic oestrogens, but more detailed investigation is needed because of the possibility of a simultaneous sulphurylation at positions 3 and 17. Any such disulphates would not be detected by the methylene blue technique since the method would only give the total amount of sulphate esters (both monosulphates and disulphates) produced.

DISCUSSION

All the sulphotransferases are eluted together from Sephadex G-200 columns either under routine preparative conditions or on a 65 cm column with a length:diameter ratio of 80:1. Since it is found that phenol sulphotransferase probably has a molecular weight around 67,000 it would be reasonable to assume that all the sulphotransferases have molecular weights lying between 60,000 and 70,000. This value should be contrasted with that of greater than 200,000 claimed by Carroll & Spencer (1964) for the sulphotransferases of rat liver.

It is clear that the synthesis of p-nitrophenyl sulphate is catalyzed by at least three enzymes, the main one being a specific phenol sulphotransferase and the other two being steroid sulphotransferases. The

phenol sulphotransferase has a pH optimum between 5.6 and 5.8 and is independent of the presence of added Mg^{2+} ions which are required by the other two enzymes having pH optima between 6.5 and 7.5. If this complex situation is typical of mammalian livers in general then the reasons for many of the inconsistencies in previous work on the enzymatic synthesis of aryl sulphates become clear. Such inconsistencies are, for instance, the requirement for Mg^{2+} ions claimed by Segal (1955) but disputed by Gregory & Lipmann (1957) or the positions of the pH optimum which have variously been reported between 6 and 8.5. The only previous mention of a dual pH optimum for the synthesis of p-nitrophenyl sulphate is that of SubbaRao, Sastry & Ganguly (1963) who reported optima at 6 and 7.5 with preparations from rat liver. Such conflicting reports are immediately explicable if more than one enzyme is involved in the synthesis of p-nitrophenyl sulphate. It is interesting to compare this situation, where several enzymes of overlapping specificity occur, to that of the synthesis of glucuronides by liver preparations where again several enzymes of different, but overlapping specificity are involved (Storey, 1965).

The role of Mg^{2+} ions in sulphotransferase activity is not clear. It should be noted that adenylyl sulphate (APS), unlike ATP, only weakly chelates Mg^{2+} ions. The values of the association constants are $24M^{-1}$ and $4500M^{-1}$ for APS and ATP respectively (Yount, Simchuk, Yu & Kottke, 1966). It can therefore probably be assumed that PAPS likewise only weakly binds Mg^{2+} ions and hence the true substrate for the sulphotransferases is likely to be free PAPS and not Mg - PAPS complex. If this is so, then it is difficult to see why the steroid sulphotransferases require added Mg^{2+} ions for maximal activity. Obviously further work will be necessary to clarify the part played by Mg^{2+} ions in sulphotransferase activity, particularly because possibility exists that Mg^{2+} ions react directly with the enzymes. If the enzymes do react directly with Mg^{2+} ions then the reasons for the observed difference in the requirement for Mg^{2+} ions by the phenol sulphotransferase and steroid sulphotransferases are simply that the phenol sulphotransferase strongly binds Mg^{2+} ions which are not removed by dialysis against EDTA, whereas the steroid sulphotransferases only weakly bind Mg^{2+} ions which are removed by dialysis against EDTA.

The elution of phenol sulphotransferase from

DEAE-Sephadex at pH 7.5 shows that the enzyme is strongly adsorbed to the anion exchanger and can only be eluted at high ionic strengths. This suggests that the enzyme is negatively charged at pH 7.5. This conclusion is also supported by the fact that the enzyme is not adsorbed on the cation exchanger, CM-Sephadex, at pH 7.5 even at a low ionic strength of 0.008, as will be seen in a later chapter. These two observations definitely indicate that the isoelectric point of the enzyme is lower than 7.5. As seen above, the isoelectric fractionation of the enzyme completely inactivated it and so the determination of isoelectric point was not achieved. The electrolysis method effectively removes all ions and thus gives the isoelectric point of uncomplexed proteins and in this way gives the isoionic point of proteins. It is therefore possible that the isoionic point of phenol sulphotransferase is lower than 6.6, the enzyme being stable above pH 6.6.

All the steroid sulphotransferases require added Mg^{2+} ions for maximal activity and it is on this account that much of the work of Boström and his group on the distribution of sulphotransferases in the various tissues is open to criticism. In these studies, they prepared PAPS by incubating appropriate

concentrations of ATP and Mg^{2+} ions in KH_2PO_4 buffer with the sulphate activating enzymes of yeast. After 30 min, they stopped the reaction by adding an excess of EDTA and used the resultant mixture containing PAPS to assay the sulphotransferases (Boström, Franksson & Wengle, 1964; Wengle, 1966). Obviously EDTA present in such PAPS preparations would compete with the steroid sulphotransferases for Mg^{2+} ions.

The present work gives the first demonstration that cholesterol can be sulphurylated by enzyme preparations from mammalian tissues. All previous attempts (Schneider & Lewbart, 1956; Nose & Lipmann, 1958; Baulieu et al., 1965) to demonstrate its synthesis in vitro failed. The reason for this failure is not obvious, but one factor could be the very limited solubility of cholesterol in aqueous media and another the rather firm binding of cholesteryl sulphate to protein which can cause very poor recoveries of the ester (Roy, 1963; Banerjee & Roy, 1967).

As pointed out in a previous chapter, it seems to have been shown that androstenolone and oestrone sulphotransferases can both accept 2-naphthylamine and p-nitrophenol as substrates. The powerful inhibition by androstenolone methyl ether of 2-naphthyl sulphamate synthesis in both androstenolone and oestrone sulphotransferases, described here, lend

support to this hypothesis. The inhibition by androstenolone methyl ether of 2-naphthyl sulphamate synthesis in guinea pig liver preparations was first noted and studied by Roy (1960a, 1961, 1964). However the quantitative data of Roy on the above inhibition must have little absolute significance because, in the light of the present work, more than one enzyme was involved.

The specificity of the steroid sulphotransferases is obscure. The sulphurylation of 3β-hydroxy groups in androstenolone, pregnenolone and cholesterol by a single enzyme is explicable; but the enzyme also seems to act on androsterone, a 3α-hydroxy steroid. Similarly the oestrone sulphotransferase fraction also catalyzes the sulphurylation of oestriol and oestradiol where the possibility of the formation of disulphates cannot be excluded. Lastly, it must be pointed out that in the present work, the products of steroid sulphurylation were not actually identified. In vivo steroids can easily undergo many reactions, including oxidation and reduction, apart from sulphurylation; but these reactions were presumably absent in the present cases, because all such reactions require cofactors of some sort, and in the reasonably purified enzymes used for specificity studies these cofactors

were absent.

With regard to the vitamin A experiments, the fact that vitamin A (retinol) or vitamin A acetate (retinyl acetate) had no effect on the sulphotransferase activity does not permit any unambiguous conclusion to be drawn about the role of vitamin A in sulphotransferase activity, because the enzyme preparations might have been replete with vitamin A. The rather high lability of the sulphotransferases towards acetone could perhaps be taken to indicate that a lipid-soluble factor is involved in their structure.

CHAPTER 5

FURTHER PURIFICATION OF PHENOL SULPHOTRANSFERASE

Introduction

It was seen earlier that DEAE-Sephadex fractionation of the sulphotransferases separates the phenol sulphotransferase completely from the steroid sulphotransferases. The usual specific activity of phenol sulphotransferase at this stage is 33, expressed as millimicromoles of p-nitrophenyl sulphate formed per 10 min per mg protein using 0.100 mM p-nitrophenol and 0.100 mM PAPS in the enzyme assays.

The enzyme is inactivated by acetone fractionation and is stable only at pH 7-7.5 in the presence of mercaptoethanol and also of EDTA if completely metal free conditions are not used. The methods of purification are therefore limited by the properties of the enzyme.

Experimental

Adsorption on Alumina C_γ Gel: An alumina C_γ gel (Colowick & Kaplan, 1955) containing 0.283% by dry weight of alumina was a gift from Dr. R.L. Blakley

of the Department of Biochemistry. This gel was used in attempts to differentially adsorb the enzyme. After each addition of the gel the mixture was allowed to stand for $\frac{1}{2}$ hr. and then briefly centrifuged. The supernatants at different concentrations of the adsorbent were then assayed.

Fractional Precipitation by $(NH_4)_2SO_4$: The details of the procedure for fractional precipitation by $(NH_4)_2SO_4$ are given earlier.

CM-Sephadex Fractionation: The enzyme was dialysed against 0.01 M Tris - acetic acid buffer, pH 7.5, containing 0.01 M mercaptoethanol under metal free conditions. It was then chromatographed on a CM-Sephadex column (1.6 x 30 cm) using a rectilinear gradient formed from 80 ml of 0.01 M Tris - acetic acid, pH 7.5, and 80 ml of 0.01 M EDTA - NaOH, pH 7.5, both buffers containing 0.01 M mercaptoethanol. 4 ml fractions were collected. The enzyme was eluted in tubes 3-5 with a peak at tube 4. This showed that even at these low ionic strengths the enzyme was not adsorbed on the column. No purification of the effluent enzyme was achieved.

Sephadex G-100 Fractionation: For fractionation on

Sephadex G-100, a column 1.8 x 82 cm was set up and equilibrated with 0.03 M EDTA - NaOH buffer, pH 7.5, containing 0.01 M mercaptoethanol. The column was developed using the same buffer. The enzyme was eluted as a sharp peak after about 100 ml of buffer had passed through the column. The fractions containing the enzyme were collected and concentrated by ultrafiltration and then dialysed against 0.03 M EDTA - NaOH buffer, pH 7.5, containing 0.01 M mercaptoethanol. The enzyme was stable for prolonged periods in the cold when kept at pH 7.5 in 0.03 M EDTA and 0.01 M mercaptoethanol.

Fractionation on Cellulose Anion Exchangers: Further purification of the enzyme was attempted by the use of two other anion exchangers, namely DEAE-cellulose and ECTEOLA cellulose. The scheme of preparation of two typical batches is described below.

Preparation 1 : In this preparation the enzyme was dialysed exhaustively against 0.01 M EDTA - NaOH buffer, pH 7.5, containing 0.1 M mercaptoethanol. About 2 ml of the enzyme was then loaded on a DEAE-cellulose column (1.6 x 30 cm). Elution was carried out with a linear concentration gradient formed from 200 ml of the above buffer and 200 ml of a buffer containing 0.03 M EDTA - 0.05 M Tris - 0.30 M sodium

acetate, pH 7.5, containing 0.1 M mercaptoethanol. The column was run at 0.2 ml/min. 4 ml fractions were collected. Under these conditions the enzyme was eluted in a broad zone between tube 25 and 50 with a peak at tube 38.

The fractions containing the enzyme were concentrated by ultrafiltration to about 2 ml and dialysed against 0.01 M EDTA - NaOH, pH 7.5, containing 0.1 M mercaptoethanol. The dialysed enzyme was then applied to a column (1.6 x 30 cm) of ECTEOLA cellulose which was run under the same conditions as the DEAE-cellulose column. As before 4 ml fractions were collected into tubes. The enzyme was eluted sharply between the 19th and the 27th tube with a peak at the 23rd tube.

All the fractions containing the enzyme were collected, concentrated by ultrafiltration to about 2 ml and dialysed against 0.01 M EDTA - NaOH, pH 7.5, containing 0.1 M mercaptoethanol. The dialysed enzyme was then rechromatographed on an identical column of ECTEOLA cellulose under the same conditions. There was a severe loss of enzyme activity which was eluted in three tubes only. This precluded any further attempts at purification of this preparation.

Preparation 2 : Another batch of phenol sulphotransferase was taken to the stage of the first ECTEOLA cellulose fractionation described above and instead of rechromatography on another ECTEOLA cellulose column, it was chromatographed on a Sephadex G-100 column (1.8 x 82 cm) equilibrated with 0.03 M EDTA - NaOH (pH 7.5) containing 0.1 M mercaptoethanol. The enzyme was eluted between tubes 21 and 25 with a peak at tube 23 but there was again an appreciable loss of enzyme activity.

Several attempts were then made to sharpen the enzyme peak during DEAE-cellulose fractionation by employing a concave gradient formed by using 100 ml of the starting buffer in each of the chambers 1,2,3,4 and using 100 ml of the limit buffer in chamber 5 of a variable gradient device (Peterson & Sober, 1959). The concentration gradient was checked by measuring the conductivity of the fractions in a Philips conductivity-measuring bridge.

However, concave gradient elution did not sharpen the enzyme peak and since the procedure was wasteful in that no protein was eluted till the 65th tube, the operation was abandoned.

The protein contents of the final enzyme solutions were measured by the refractometric method

(Cecil & Ogston, 1951).

Results

Alumina C_γ Gel: Aliquots of the enzyme were brought to concentrations of 0.06, 0.10 and 0.17% Al_2O_3 by adding the requisite amounts of alumina C_γ gel. In each case the activity of the supernatant was measured. The results expressed relative to those of the control enzyme are tabulated below.

Enzyme sample	Al_2O_3 concentration %	Activity in the supernatant as % of control	Specific activity relative to control
Control	-	100	1.00
1	0.06	95	1.16
2	0.10	78	0.85
3	0.17	14	0.23

The results suggested that no great purification could be achieved by this method.

$\underline{(NH_4)_2SO_4 \text{ Fractionation}}$. The results of fractional precipitation with $(NH_4)_2SO_4$ are presented below.

Enzyme sample	Precipitated between $(NH_4)_2SO_4$ saturation	Recovery of activity as % control	Specific activity relative to control
Control	-	100	1.00
1	0 - 0.3	25	1.54
2	0.3 - 0.4	25	0.57
3	0.4 - 0.5	21	0.51
4	0.5 - 0.6	2	0.11
5	0.6 - 0.7	0	0

The results showed that no significant purification could be achieved by $(NH_4)_2SO_4$ fractionation.

Sephadex G-100 Fractionation. Fractionation on Sephadex G-100 usually gave a 3 fold purification with about 70% recovery of enzyme activity. The usual specific activity at the end of Sephadex G-100 fractionation was 110 millimicromoles of p-nitrophenyl sulphate formed per 10 min per mg protein at 0.100 mM p-nitrophenol and 0.100 mM PAPS.

Chromatography on Cellulose Anion Exchanger. The yield and specific activity at the different stages of the purification of preparations 1 and 2 are presented in Table 8. It is seen that the maximum specific activity obtained during purification was about 350 millimicromoles of p-nitrophenyl sulphate formed per 10 min per mg protein at 0.100 mM p-nitrophenol and 0.100 mM PAPS and purification beyond this was not achieved because of enzyme losses.

(TABLE 8 - next page)

Table 8

Total yield and specific activity of phenol sulphotransferase at the different stages of purification. The yields are expressed as millimicromoles of p-nitrophenyl sulphate formed in 10 min and the specific activities as millimicromoles of p-nitrophenyl sulphate formed per 10 min per mg protein (based on refractive index) both at 0.100 mM PAPS and 0.100 mM p-nitrophenol.

Stage	Yield	Specific Activity
Preparation 1		
After DEAE-Sephadex	1607	35.7
After DEAE-Cellulose	1452	167
After 1st ECTEOLA Cellulose	927	352
After 2nd ECTEOLA Cellulose	215	89.8
Preparation 2		
After DEAE-Sephadex	1273	31.6
After DEAE-Cellulose	908	147
After ECTEOLA Cellulose	539	311
After Sephadex G-100	288	282

DISCUSSION

The treatment of the enzyme with alumina C_Y gel at an Al_2O_3 concentration of 0.06% was conveniently incorporated at an early stage of enzyme purification, that is just following the pH 5 precipitation of the sulphotransferases (Chapter 3).

Chromatography on Sephadex G-100 was routinely used to follow the separation of phenol sulphotransferase on DEAE-Sephadex. Although the enzyme was not as purified as hoped, it was quite stable and was found to be free from PAPS-degrading activity even when tested under the extreme conditions used by Jones; that is at an effective enzyme concentration of nearly 10 times that used in the normal assay. The enzyme was therefore suitable for kinetic studies.

CHAPTER 6

BIREACTANT ENZYME KINETICS

The reaction catalyzed by phenol sulphotransferase utilizing p-nitrophenol as the sulphate acceptor and PAPS as the sulphate donor can be represented by

$$\underset{\text{(p-nitrophenol)}}{\text{NP}} + \text{PAPS} \rightleftharpoons \underset{\text{(p-nitrophenyl sulphate)}}{\text{NPS}} + \text{PAP}$$

This is a case in which two reactants give two products under the catalytic influence of an enzyme. The reaction is therefore bireactant in both directions since it involves two kinetically significant substrates and two kinetically significant products. In the nomenclature of Cleland (1963a) the reaction is bi bi, that is, bireactant in both the forward and backward directions. The scope of the present discussion will be restricted only to the basic bi bi reaction mechanisms one of which, as will be seen in a later chapter, is applicable to the phenol sulphotransferase reaction. Cleland (1963a) has proposed a systematic nomenclature for describing enzymatic reaction mechanisms and has derived the full rate equations for a large number of reaction

mechanisms using the schematic method of King & Altman (1956). The main advantages of having a full rate equation for the entire mechanism in hand are that the equations can be directly simplified to give the initial velocity equations simply by setting all product concentrations to zero and that product inhibition equations can be readily derived by substituting zero for all products except the product under study. Further the full rate equation describes the steady state kinetic behaviour of the system at any time and at any point, whether near or far from equilibrium.

Reactions are divided into two main classes "sequential" and nonsequential or "ping pong" (Cleland, 1963a). In the sequential mechanism all substrates must add to the enzyme before any products are released. In the ping pong mechanism, on the other hand, one or more products are released before all substrates have added to the enzyme so that the enzyme exists in two or more stable forms between which it oscillates during the reaction. The sequential mechanism is "ordered" if there is a compulsory order of addition of substrates and release of products and "random" if no such compulsory pathways exist.

Restricting the discussion to bi bi reactions there is a special case of the ordered reaction in which no central ternary complex is formed at all or its concentration is kinetically insignificant and this special case of the ordered sequential mechanism is called the Theorell-Chance mechanism (Theorell & Chance, 1951).

INITIAL VELOCITY EQUATIONS

The initial velocity equations for the basic bi bi reactions are presented below. The mechanisms are graphically depicted by the short-hand method of Cleland (1963a). The enzyme is represented by a horizontal line and substrate additions and product dissociations are represented by arrows pointing towards and away from the horizontal line respectively.

A, B are the two substrates and C, D are the two products and E is the free enzyme. The initial velocity equations given here are the simplified forms of the full rate equations given by Cleland (1963a) and are derived by substituting zero for the product concentrations. The concentrations of A, B, C, D are represented by a, b, c, d respectively. The initial velocity is represented by v and the maximum

initial velocity by V. K_{ma} and K_{mb} are the limiting Michaelis constants for A and B respectively, that is, Michaelis constant for A with B saturating and Michaelis constant for B with A saturating respectively. K_a and K_b are the dissociation constants for the EA and EB complexes respectively.

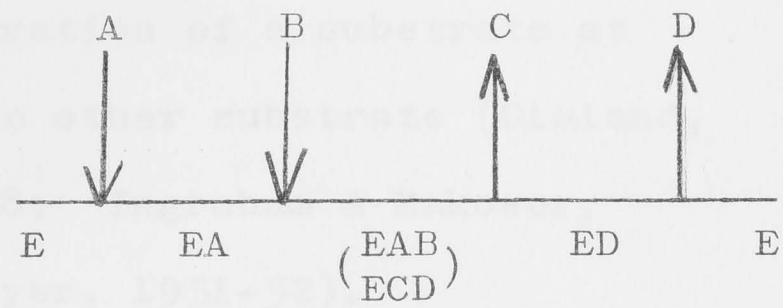
It is immediately clear that the symbols used in the present work differ from those of Cleland (1963a) as shown below.:-

	Cleland	This work
Michaelis constant for A, B saturating	K_a	K_{ma}
Michaelis constant for B, A saturating	K_b	K_{mb}
$\frac{(E)(A)}{(EA)}$	+ Usually K_{ia}	K_a
$\frac{(E)(B)}{(EB)}$	+ Usually K_{ib}	K_b

+ The K_{ia} and K_{ib} terms of Cleland are inhibition constants and are not necessarily the dissociation constants for the EA and EB complexes, for example in an ordered reaction EB complex may not be formed at all, but there is still a K_{ib} term which then has a different meaning and merely represents a group of various kinetic constants.

A. Sequential Mechanism

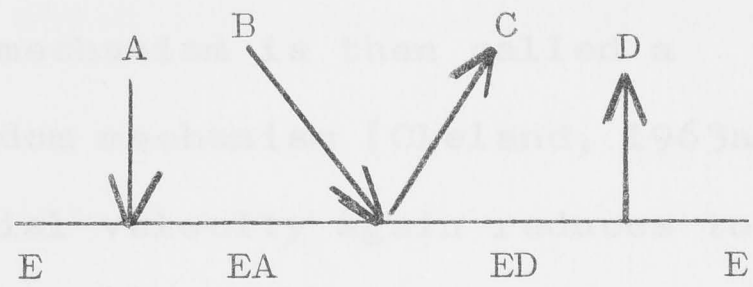
(a) Ordered



The initial velocity equation is given by

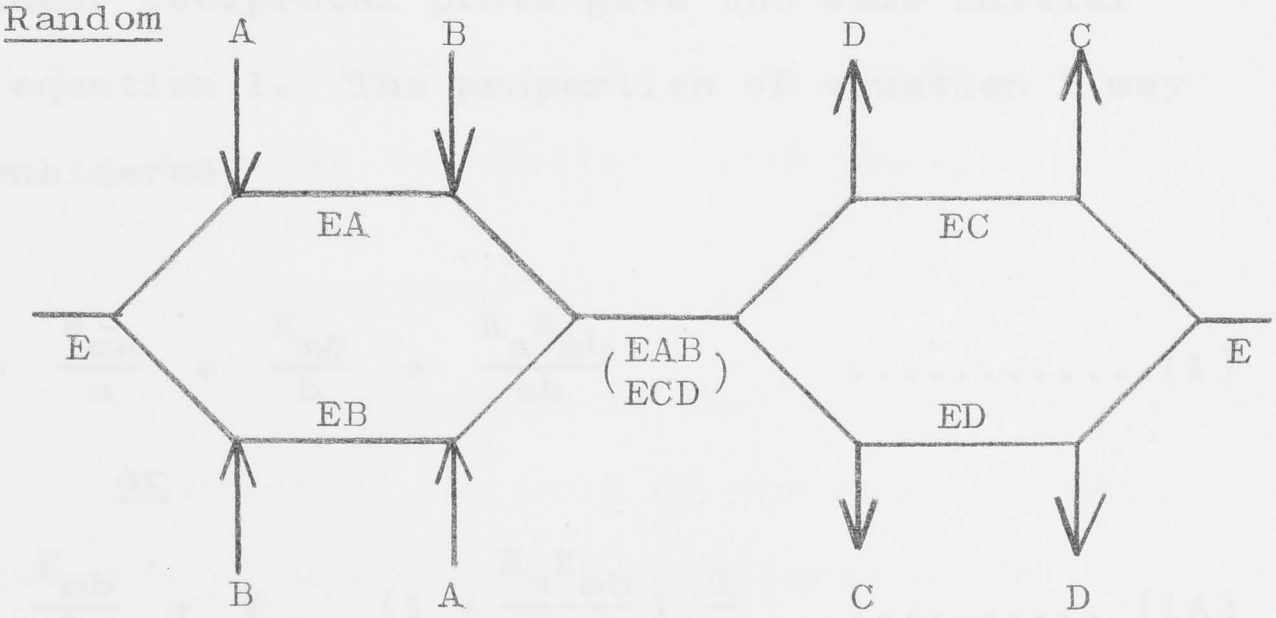
$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} \quad \ldots\ldots\ldots\ldots (1)$$

Theorell-Chance



The initial velocity equation is the same as 1.

(b) Random



Steady state treatment of a general random reaction gives a complex rate equation containing squared terms. The equation does not give linear

relationship between reciprocal initial velocity and reciprocal initial concentration of a substrate at nonsaturating levels of the other substrate (Cleland, 1963a; Alberty, 1953, 1958; Ingraham & Makower, 1954; Segal, Kachmar & Boyer, 1951-52).

If in the above mechanism the interconversion of EAB and ECD complexes is slow in comparison to all the other steps, then it can be assumed that all the steps other than the interconversion are essentially in equilibrium. The mechanism is then called a rapid equilibrium random mechanism (Cleland, 1963a). In this case the initial velocity again reduces to equation 1.

Thus all the above sequential mechanisms giving linear reciprocal plots give the same initial velocity equation 1. The properties of equation 1 may now be considered.

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} \qquad \ldots\ldots\ldots\ldots (1)$$

or

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b} + K_{ma}\left(1 + \frac{K_a K_{mb}}{K_{ma} b}\right)\frac{1}{a} \qquad \ldots\ldots\ldots (1A)$$

also

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + K_{mb}\left(1 + \frac{K_a}{a}\right)\frac{1}{b} \qquad \ldots\ldots\ldots (1B)$$

From equation 1A it is seen that when $\frac{1}{v}$ is plotted against $\frac{1}{a}$ at fixed b, a series of straight lines will be obtained with slope $= K_{ma}\left(1 + \frac{K_a K_{mb}}{K_{ma} b}\right)\frac{1}{V}$ and vertical intercept $= \left(1 + \frac{K_{mb}}{b}\right)\frac{1}{V}$.

It is derived below that all such straight lines will intersect to the left of the ordinate and the coordinates of the point of intersection are $-\frac{1}{K_a}$, $\frac{1}{V}\left(\frac{K_a - K_{ma}}{K_a}\right)$ which are independent of both a and b.

Hence the point of intersection will lie above, below or on the abscissa depending on whether $K_a \gtrless K_{ma}$, $K_a < K_{ma}$ or $K_a = K_{ma}$ respectively.

From equation 1B it will also be seen that when $\frac{1}{v}$ is plotted against $\frac{1}{b}$ at fixed a, another series of straight lines will be obtained with slope $= K_{mb}\left(1 + \frac{K_a}{a}\right)\frac{1}{V}$ and vertical intercept $= \left(1 + \frac{K_{ma}}{a}\right)\frac{1}{V}$.

It is also derived below that all such straight lines will intersect to the left of the ordinate and the coordinates of the point of intersection are

$-\frac{K_{ma}}{K_a K_{mb}}$, $\frac{1}{V}\left(\frac{K_a - K_{ma}}{K_a}\right)$ which are independent of both a and b. Again the point of intersection will lie above, below or on the abscissa depending on whether $K_a \gtrless K_{ma}$, $K_a \lessgtr K_{ma}$ or $K_a = K_{ma}$ respectively.

The points of intersection of the straight lines governed by equation 1 can be readily obtained.

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} \qquad \ldots\ldots\ldots\ldots(1)$$

At fixed b and variable a, let us take two fixed concentrations of b, namely b_1 and b_2. The straight lines will intersect at a definite $\frac{1}{v}$.

$$\therefore \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b_1} + \frac{K_a K_{mb}}{ab_1} =$$

$$1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b_2} + \frac{K_a K_{mb}}{ab_2}$$

$$\text{or } K_{mb}\left(\frac{1}{b_1} - \frac{1}{b_2}\right) = \frac{K_a K_{mb}}{a}\left(\frac{1}{b_2} - \frac{1}{b_1}\right)$$

$$\therefore \frac{1}{a} = -\frac{1}{K_a}$$

Substituting $K_a = -a$ in 1 with $b = b_1$

$$\frac{V}{v} = 1 - \frac{K_{ma}}{K_a} + \frac{K_{mb}}{b_1} - \frac{K_{mb}}{b_1}$$

$$\therefore \frac{1}{v} = \frac{1}{V} \left(\frac{K_a - K_{ma}}{K_a} \right) .$$

It follows that the coordinates of the point of intersection are

$$- \frac{1}{K_a} , \frac{1}{V} \left(\frac{K_a - K_{ma}}{K_a} \right)$$

At variable b and fixed a, as before taking two fixed concentrations a_1 and a_2 and setting $\frac{1}{v}$ = constant

$$1 + \frac{K_{ma}}{a_1} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{a_1 b} =$$

$$1 + \frac{K_{ma}}{a_2} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{a_2 b}$$

$$\underline{\text{or}} \quad K_{ma} \left(\frac{1}{a_1} - \frac{1}{a_2} \right) = \frac{K_a K_{mb}}{b} \left(\frac{1}{a_2} - \frac{1}{a_1} \right)$$

$$\therefore \frac{1}{b} = - \frac{K_{ma}}{K_a K_{mb}}$$

Substituting the above value in one of the above equations

$$1 + \frac{K_{ma}}{a_1} - \frac{K_{ma}}{K_a} - \frac{K_{ma}}{a_1} = \frac{V}{v}$$

$$\therefore \quad \frac{1}{v} = \frac{1}{V} \left(\frac{K_a - K_{ma}}{K_a} \right)$$

The coordinates of the point of intersection are therefore

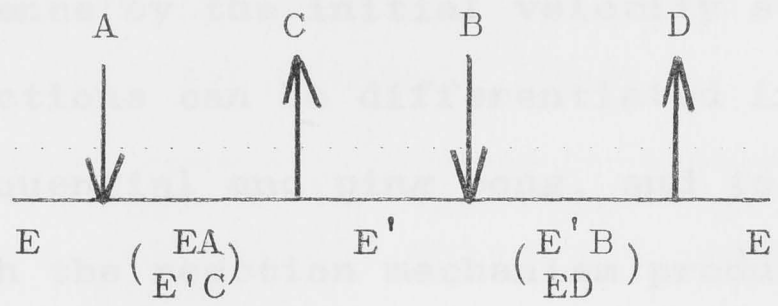
$$- \frac{K_{ma}}{K_a K_{mb}} \quad , \quad \frac{1}{V} \left(\frac{K_a - K_{ma}}{K_a} \right) .$$

For the special case in which $K_a = K_{ma}$, equation 1 reduces to

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_{ma} K_{mb}}{ab} \qquad \ldots\ldots\ldots\ldots (2)$$

and as discussed above the plots of $\frac{1}{v}$ against $\frac{1}{a}$ or $\frac{1}{b}$ at fixed b or a respectively then yield two families of straight lines, both intersecting on the abscissa, the horizontal intercepts giving $- \frac{1}{K_{ma}}$ and $- \frac{1}{K_{mb}}$ respectively.

B. Ping Pong Mechanism



Where E and E' are the two stable enzyme forms between which the enzyme oscillates during the reaction.

The initial velocity in this case is given by

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} \qquad \text{.....(3)}$$

In equation 3 the last terms of equations 1 and 2 are missing.

Equation 3 can be rewritten as

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b} + K_{ma} \cdot \frac{1}{a} \qquad \text{.....(3A)}$$

$$\text{and } \frac{V}{v} = 1 + \frac{K_{ma}}{a} + K_{mb} \cdot \frac{1}{b} \qquad \text{.....(3B)}$$

Equation 3A shows that the slope ($= \frac{K_{ma}}{V}$) of the $\frac{1}{v}/\frac{1}{a}$ plot at fixed b is constant and equation 3B shows that the slope ($= \frac{K_{mb}}{V}$) of the $\frac{1}{v} / \frac{1}{b}$ plot at fixed a is constant.

The reciprocal plots therefore give a series of parallel straight lines with both variable a- fixed b

and variable b - fixed a experiments.

Hence by the initial velocity studies alone enzyme reactions can be differentiated into two groups, sequential and ping pong, and to further distinguish the reaction mechanism product inhibition studies are generally carried out.

PRODUCT INHIBITION STUDIES

These are carried out by studying the effect of products, added one at a time, on the initial velocity of an enzymatic reaction.

From the full rate equations of the various enzymatic mechanisms, Cleland (1963a, b) derived the product inhibition equations and patterns for a number of bi bi reactions and the inhibition patterns for the basic bi bi mechanisms are presented in Table 9 below. It is then apparent that all the mechanisms given in Table 9 are unambiguously distinguished by initial velocity studies coupled with product inhibition results.

Table 9

Product inhibition patterns for bi bi mechanisms.
(After Cleland, 1963a).

Mechanism	Non-saturating fixed substrate	Variable substrate	Added product	Inhibition type
Sequential				
Ordered	A	B	C	NC
			D	NC
	B	A	C	NC
			D	Comp.
Theorell-Chance	A	B	C	Comp.
			D	NC
	B	A	C	NC
			D	Comp.
Rapid equil. random	A	B	C	Comp.
			D	Comp.
	B	A	C	Comp.
			D	Comp.
Rapid equil. random + dead end EAC complex	A	B	C	Comp.
			D	Comp.
	B	A	C	NC
			D	Comp.
Random	A	B	C	NC + +
			D	NC
	B	A	C	NC +
			D	NC +
Nonsequential				
Ping pong	A	B	C	Comp.
			D	NC
	B	A	C	NC
			D	Comp.

NC = Noncompetitive
Comp. = Competitive
NC + = Reciprocal plots are non linear. Vertical intercepts are hyperbolic functions of inhibitor while slopes are a more complex function of inhibitor.

Of all the mechanisms discussed above the random bi bi mechanism is the most general. The steady state treatment of this mechanism leads to complex rate equations containing squared terms as mentioned earlier. This mechanism however provides for the possibility that the interconversion of the central ternary complexes is so slow that the other steps leading to their formation may be considered to be essentially in equilibrium and the initial velocity equation then reduces to equation 1 (Alberty, 1953, 1956, 1958). This mechanism is called the rapid equilibrium random mechanism (Cleland, 1963a). It is therefore obvious that of all the mechanisms giving the initial velocity equation 1, the rapid equilibrium random mechanism is the most general as it does not imply a definite order of addition of substrates and of release of products.

The rapid equilibrium random mechanism is therefore treated in detail below and a complete algebraic solution of the initial velocity of the mechanism is given. The algebraic treatment has been extended to cover product inhibition and the possible formation of dead end complexes. It will be seen later that phenol sulphotransferase acts by a rapid equilibrium random mechanism with the formation of one dead end complex.

ALGEBRAIC SOLUTION OF RAPID EQUILIBRIUM RANDOM MECHANISM

Let the reaction be represented by

$$E + A + B \rightleftharpoons E + C + D$$

where E is the free enzyme, A and B are the two substrates and C and D are the two products. Let a, b, c and d be the concentrations of A, B, C and D respectively. The case in which D can only combine with the free enzyme and C can combine both with the free enzyme and an enzyme-substrate complex to form a dead end ternary complex will be considered. The cases where C cannot form a dead end complex or D can also form a dead end complex are easily covered by the treatment.

The formation of a dead end EAC complex can be represented by the following

$$EA + C \underset{}{\overset{K_1}{\rightleftharpoons}} EAC \overset{K_2}{\rightleftharpoons} EC + A$$

where $K_1 = \frac{(EA)c}{(EAC)}$

and $K_2 = \frac{(EC)a}{(EAC)}$

It is assumed that the rate of formation and dissociation of such inactive complexes is much greater than the interconversion of the active EAB and ECD

complexes. As before the dissociation constants for the EC and ED complexes are represented by K_c and K_d respectively.

Using the symbols used in this chapter, the full rate equation given by Cleland (1963a,b,c) for the mechanism in which only one inactive ternary complex EAC is formed can be written down as

$$v = \frac{abV - cd\,V' \dfrac{K_a K_{mb}}{K_{mc} K_d}}{ab + bK_{ma} + aK_{mb} + K_a K_{mb} + \dfrac{K_a K_{mb} d}{K_d} + \dfrac{K_a K_{mb} c}{K_c} + \dfrac{K_a K_{mb} cd}{K_{mc} K_d} + \dfrac{K_{mb} ac}{K_l}} \qquad \ldots\ldots\ldots\ldots(4)$$

where V' = maximum initial velocity in the reverse direction.

Initial Velocity Equations

Let the following be envisaged

$$\left.\begin{array}{lcll} E + A & \rightleftharpoons & EA & \\ E + B & \rightleftharpoons & EB & \\ EA + B & \rightleftharpoons & EAB & \\ EB + A & \rightleftharpoons & EAB & \end{array}\right\} \xrightarrow{k} \text{Products}$$

Since the initial velocity is studied the product concentration is zero.

$$\text{Let} \quad K_a = \frac{(E)a}{(EA)}$$

$$K_b = \frac{(E)b}{(EB)}$$

$$K_{mb} = \frac{(EA)b}{(EAB)}$$

$$K_{ma} = \frac{(EB)a}{(EAB)}$$

K_{ma} is not independent but is related to the other constants by $K_{ma} = \frac{K_a K_{mb}}{K_b}$

From the conservation of enzyme, E = free enzyme = $E_o - (EA) - (EB) - (EAB)$

where E_o = total enzyme added.

From above $K_a = \frac{(E)a}{(EA)}$

$$\therefore \quad K_a = \frac{\left[E_o - (EA) - (EB) - (EAB)\right]a}{(EA)}$$

$$= \frac{\left[E_o - \frac{K_{mb}(EAB)}{b} - \frac{K_{ma}(EAB)}{a} - (EAB)\right]a}{\frac{K_{mb}(EAB)}{b}}$$

whence $K_a = \left[\frac{E_o b}{K_{mb}(EAB)} - 1 - \frac{K_{ma} b}{K_{mb} a} - \frac{b}{K_{mb}}\right] a$

$$\therefore (EAB) = \frac{E_o b}{\frac{K_a K_{mb}}{a} + K_{mb} + \frac{K_{ma} b}{a} + b}$$

$$\text{or} \quad (EAB) = \frac{E_o}{\frac{K_a K_{mb}}{ab} + \frac{K_{mb}}{b} + \frac{K_{ma}}{a} + 1}$$

Now v = initial velocity = k (EAB)

$$\therefore v = \frac{k E_o}{1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab}}$$

Now $k E_o = V$ = maximum velocity when all the enzyme is in the form of EAB.

$$\therefore \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} \quad \ldots\ldots\ldots(1)$$

In this case however $K_a K_{mb} = K_b K_{ma}$

$$\therefore \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_b K_{ma}}{ab} \quad \ldots\ldots\ldots(1')$$

Equations 1 and 1' can be obtained directly from equation 4 by setting c = d = o .

Rearranging 1', $\frac{V}{v} = 1 + \frac{K_{mb}}{b} + K_{ma}\left(1 + \frac{K_b}{b}\right)\frac{1}{a}$ .....(1A')

Rearranging 1, $\frac{V}{v} = 1 + \frac{K_{ma}}{a} + K_{mb}\left(1 + \frac{K_a}{a}\right)\frac{1}{b}$

.....(1B)

If the combination of B with the enzyme is not affected by the presence of A on the enzyme and vice versa, then

$$K_a = K_{ma}$$

and $K_b = K_{mb}$

Then both the equations reduce to

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_{ma}K_{mb}}{ab} \quad .....(2)$$

giving $\frac{V}{v} = 1 + \frac{K_{mb}}{b} + K_{ma}\left(1 + \frac{K_{mb}}{b}\right)\frac{1}{a}$ .....(2A)

and $\frac{V}{v} = 1 + \frac{K_{ma}}{a} + K_{mb}\left(1 + \frac{K_{ma}}{a}\right)\frac{1}{b}$ .....(2B)

Product Inhibition Equations

With added D

First let us consider that there is no dead end complexes formed and D only combines with the free enzyme.

Then
$$\begin{array}{lcl} E + A & \rightleftharpoons & EA \\ E + B & \rightleftharpoons & EB \\ EA + B & \rightleftharpoons & EAB \\ EB + A & \rightleftharpoons & EAB \\ E + D & \rightleftharpoons & ED \end{array} \xrightarrow{\quad k \quad} \text{Products}$$

As before
$$K_a = \frac{(E)a}{(EA)}$$
$$K_b = \frac{(E)b}{(EB)}$$
$$K_{mb} = \frac{(EA)b}{(EAB)}$$
$$K_{ma} = \frac{(EB)a}{(EAB)}$$
$$K_d = \frac{(E)d}{(ED)}$$

$$\text{Now } K_d = \frac{(E)d}{(ED)} = \frac{\left[E_o - (EA) - (EB) - (EAB) - (ED)\right] d}{(ED)}$$

$$\therefore (ED) = \frac{E_o - (EA) - (EB) - (EAB)}{\left(1 + \frac{K_d}{d}\right)}$$

$$\frac{K_a}{a} = \frac{(E)}{(EA)} = \frac{E_o - (EA) - (EB) - (EAB) - \dfrac{E_o - (EA) - (EB) - (EAB)}{1 + \dfrac{K_d}{d}}}{(EA)}$$

$$\text{or } \frac{K_a}{a} = \frac{\frac{K_d}{d}\left[E_o - (EA) - (EB) - (EAB)\right]}{(EA)\left(1 + \frac{K_d}{d}\right)}$$

$$\therefore \frac{K_a}{a} = \frac{\frac{K_d}{d}\left[E_o - \frac{K_{mb}(EAB)}{b} - \frac{K_{ma}(EAB)}{a} - (EAB)\right]}{\frac{K_{mb}(EAB)}{b}\left(1 + \frac{K_d}{d}\right)}$$

$$\text{or } \frac{E_o b}{K_{mb}(EAB)} = \frac{K_a \cdot d}{aK_d}\left(1 + \frac{K_d}{d}\right) + 1 + \frac{K_{ma} b}{K_{mb} a} + \frac{b}{K_{mb}} .$$

$$\text{Whence, } (EAB) = \frac{E_o}{\frac{K_a K_{mb} d\left(1 + \frac{K_d}{d}\right)}{a K_d b} + \frac{K_{mb}}{b} + \frac{K_{ma}}{a} + 1}$$

$$\therefore (EAB) = \frac{E_o}{1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab}\left(1 + \frac{d}{K_d}\right)}$$

$$\therefore v = k(EAB) = \frac{k E_o}{1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab}\left(1 + \frac{d}{K_d}\right)}$$

$$\therefore \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} \left(1 + \frac{d}{K_d}\right) \quad \ldots\ldots\ldots (5)$$

Since $K_a K_{mb} = K_b K_{ma}$, equation 5 can be rewritten as

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_b K_{ma}}{ab} \left(1 + \frac{d}{K_d}\right) \quad \ldots\ldots\ldots (5')$$

Equations 5 and 5' can again be obtained from equation 4 by setting c = o.

For variable a and fixed b, equation 5' is rewritten as

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b} + \frac{K_{ma}}{a}\left[1 + \frac{K_b}{b}\left(1 + \frac{d}{K_d}\right)\right] \quad \ldots\ldots\ldots (5A)$$

And for variable b and fixed a, equation 5 is rewritten as

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b}\left[1 + \frac{K_a}{a}\left(1 + \frac{d}{K_d}\right)\right] \quad \ldots\ldots\ldots (5B)$$

If, as before $K_a = K_{ma}$ and $K_b = K_{mb}$ then equations 5A and 5B reduce to

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b} + \frac{K_{ma}}{a}\left[1 + \frac{K_{mb}}{b}\left(1 + \frac{d}{K_d}\right)\right] \quad \ldots\ldots\ldots (6A)$$

$$\text{and } \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b}\left[1 + \frac{K_{ma}}{a}\left(1 + \frac{d}{K_d}\right)\right] \quad \text{.....(6B)}$$

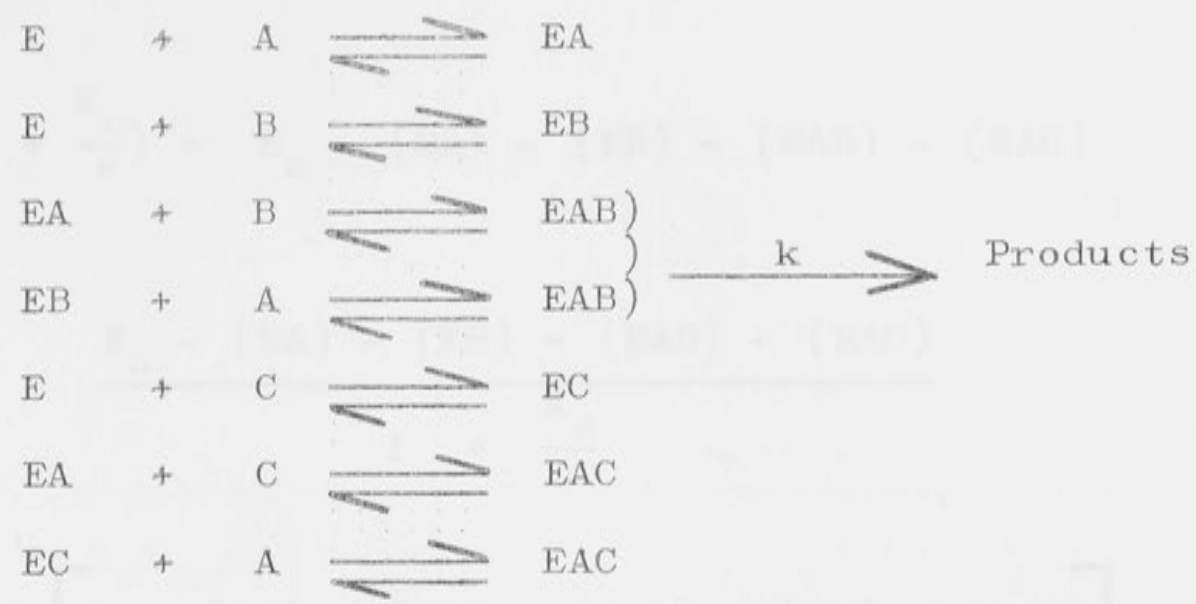
Inspection of equations 5A, 5B, 6A and 6B will immediately show that added D will give a linear competitive inhibition with respect to A at a fixed nonsaturating level of B and also a linear competitive inhibition with respect to B at a fixed nonsaturating level of A. The inhibitions are abolished at saturating concentrations of the fixed substrates since the inhibitor terms then vanish.

Similarly if C reacts in the same way as D it will exhibit identical inhibition characteristics. If, on the other hand, C can also form a dead end ternary complex by reacting with an enzyme-substrate complex then the situation is altered.

This case is treated below.

With added C

The case where C can react to also form a dead end ternary complex by combining with an enzyme-substrate complex can be represented by the following reactions.:-



As before

$$K_a = \frac{(E)a}{(EA)}$$

$$K_b = \frac{(E)b}{(EB)}$$

$$K_{mb} = \frac{(EA)b}{(EAB)}$$

$$K_{ma} = \frac{(EB)a}{(EAB)}$$

$$K_c = \frac{(E)c}{(EC)}$$

$$K_1 = \frac{(EA)c}{(EAC)}$$

$$K_2 = \frac{(EC)a}{(EAC)}$$ which is not independent

but is related to K_a, K_c and K_1 by $K_2 = K_1 \frac{K_a}{K_c}$

$$\text{Now } (EC) = \frac{(E)c}{K_c} = \frac{\left[E_o-(EA)-(EB)-(EAB)-(EC)-(EAC)\right]\ c}{K_c}$$

$$\therefore (EC)\left(1 + \frac{K_c}{c}\right) = E_o - (EA) - (EB) - (EAB) - (EAC)$$

$$\text{Or} \quad (EC) = \frac{E_o - (EA) - (EB) - (EAB) - (EAC)}{1 + \frac{K_c}{c}}$$

$$\text{Now } K_a = \frac{\left[E_o - (EA) - (EB) - (EAB) - (EC) - (EAC)\right] a}{(EA)}$$

Substituting the value of (EC) as above and simplifying

$$K_a = \frac{\frac{K_c}{c}\left[E_o - (EA) - (EB) - (EAB) - (EAC)\right] a}{(EA)\ (1 + \frac{K_c}{c})}$$

$$\therefore K_a = \frac{\frac{K_c}{c}\left[E_o - \frac{K_{mb}(EAB)}{b} - \frac{K_{ma}(EAB)}{a} - (EAB) - \frac{K_{mb}(EAB)c}{K_1 b}\right] a}{\frac{K_{mb}(EAB)}{b}\ (1 + \frac{K_c}{c})}$$

$$\text{or } K_a = \frac{\frac{K_c}{c}\left[\frac{E_o b}{K_{mb}(EAB)} - 1 - \frac{K_{ma}}{K_{mb}}\frac{b}{a} - \frac{b}{K_{mb}} - \frac{c}{K_1}\right] a}{1 + \frac{K_c}{c}}$$

$$\therefore \frac{E_o b}{K_{mb}(EAB)} = \frac{K_a c\left(1 + \frac{K_c}{c}\right)}{aK_c} + 1 + \frac{K_{ma} b}{K_{mb} a} + \frac{b}{K_{mb}} + \frac{c}{K_1}$$

$$\therefore (EAB) = \frac{E_o b}{\frac{K_a K_{mb} c\left(1 + \frac{K_c}{c}\right)}{aK_c} + K_{mb} + \frac{K_{ma} b}{a} + b + \frac{K_{mb} c}{K_1}}$$

$$\therefore v = k(EAB) = \frac{k\, E_o\, ab}{ab + bK_{ma} + a\, K_{mb} + \frac{K_a K_{mb} c\left(1 + \frac{K_c}{c}\right)}{K_c} + \frac{K_{mb} ac}{K_1}}$$

$$\therefore v = \frac{V}{1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb} c\left(1 + \frac{K_c}{c}\right)}{ab\, K_c} + \frac{K_{mb} c}{K_1 b}}$$

Whence $$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_a K_{mb}}{ab} + \frac{K_a K_{mb} c}{ab\, K_c} + \frac{K_{mb} c}{K_1 b}$$

.....(7)

Since $K_a K_{mb} = K_{ma} K_b$

Also $\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b} + \frac{K_b K_{ma}}{ab} + \frac{K_b K_{ma} c}{ab\ K_c} + \frac{K_{mb} c}{K_1 b}$

.....(7')

Equations 7 and 7' can again be directly obtained from equation 4 by setting d = o.

∴ For variable a and fixed b, equation 7' can be rewritten as

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_1}\right) + \frac{K_{ma}}{a}\left[1 + \frac{K_b}{b}\left(1 + \frac{c}{K_c}\right)\right]$$

..... (7A)

For variable b and fixed a, equation 7 can be rearranged as

$$\frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b}\left[1 + \frac{K_a}{a}\left(1 + \frac{c}{K_c}\right) + \frac{c}{K_1}\right]$$

..... (7B)

From equation 7A it can be seen that at nonsaturating b, the inhibition by C is noncompetitive with respect to A and the slopes and vertical intercepts will vary linearly with c. At saturating b, the inhibition is abolished since the terms containing c vanish.

From equation 7B it can be seen that with variable b, C will exhibit linear competitive inhibition irrespective of a.

If now, as before, $K_a = K_{ma}$ and $K_b = K_{mb}$ then equations 7A and 7B reduce to

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_1}\right) + \frac{K_{ma}}{a}\left[1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_c}\right)\right] \quad \ldots\ldots\ldots\ldots (8A)$$

$$\text{and } \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b}\left[1 + \frac{K_{ma}}{a}\left(1 + \frac{c}{K_c}\right) + \frac{c}{K_1}\right] \quad \ldots\ldots\ldots\ldots (8B)$$

Further, if $K_1 = K_c$, then equations 8A and 8B become

$$\frac{V}{v} = 1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_c}\right) + \frac{K_{ma}}{a}\left[1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_c}\right)\right] \quad \ldots\ldots\ldots\ldots (9A)$$

$$\text{and } \frac{V}{v} = 1 + \frac{K_{ma}}{a} + \frac{K_{mb}}{b}\left[1 + \frac{K_{ma}}{a}\left(1 + \frac{c}{K_c}\right) + \frac{c}{K_c}\right] \quad \ldots\ldots\ldots\ldots (9B)$$

The inhibition patterns as mentioned above are obeyed.

Similarly, if D reacts in the same way as C identical treatment can be followed for D as well and the equations will then give the inhibition characteristics in a rapid equilibrium random mechanism in which

two dead end complexes, EAC and EBD, can form.

Calculation of Inhibition Constants

The true inhibition constants can be calculated from the apparent inhibition constants as shown below. Let K_i' be the apparent inhibition constants.

Calculation of K_d. K_i' is obtained by replotting the slope of equation 5A with b fixed - a variable against d. The horizontal intercept then gives K_i'.

$\therefore$ From equation 5A

$$K_{ma}\left[1 + \frac{K_b}{b}\left(1 + \frac{d}{K_d}\right)\right] = 0$$

or $$\frac{K_b}{b}\left(1 + \frac{d}{K_d}\right) = -1$$

$$\therefore \quad 1 + \frac{d}{K_d} = -\frac{b}{K_b}$$

From definition $K_i' = -d =$ horizontal intercept

$$\therefore \quad \frac{K_i'}{K_d} = 1 + \frac{b}{K_b} = \frac{b + K_b}{K_b}$$

$$\therefore \quad K_d = K_i' \frac{K_b}{K_b + b} \qquad \ldots\ldots\ldots (10A)$$

Also with a fixed - b variable, equation 5B gives

$$K_{mb}\left[1 + \frac{K_a}{a}\left(1 + \frac{d}{K_d}\right)\right] = 0$$

$$\therefore \quad \frac{K_a}{a}\left(1 + \frac{d}{K_d}\right) = -1$$

$$\therefore \quad \frac{d}{K_d} = -\left(1 + \frac{a}{K_a}\right)$$

Noting that $K_i' = -d$,

$$K_d = K_i' \frac{K_a}{K_a + a} \qquad \ldots\ldots\ldots (10B)$$

If now $K_a = K_{ma}$ and $K_b = K_{mb}$, equations 10A and 10B become

$$K_d = K_i' \frac{K_{mb}}{K_{mb} + b} \qquad \ldots\ldots\ldots (11A)$$

and $$K_d = K_i' \frac{K_{ma}}{K_{ma} + a} \qquad \ldots\ldots\ldots (11B)$$

Calculation of K_c and K_1. From equation 7A, K_c is calculated from the K_i' (slope) and K_1 is calculated from the K_i' (vertical intercept).

From the slope of equation 7A with b fixed - a variable

$$1 + \frac{K_b}{b}\left(1 + \frac{c}{K_c}\right) = 0$$

$$\therefore \quad 1 + \frac{c}{K_c} = -\frac{b}{K_b}$$

$$\text{or} \quad \frac{c}{K_c} = -\left(1 + \frac{b}{K_b}\right)$$

Again by definition $K_i' = -c$ = horizontal intercept

$$\therefore \quad K_c = K_i' \frac{K_b}{K_b + b} \qquad \ldots\ldots\ldots (12A)$$

From the replot of the vertical intercepts of equation 7A with b fixed - a variable against c, K_i' is obtained from the horizontal intercept.

$$\therefore \quad 1 + \frac{K_{mb}}{b}\left(1 + \frac{c}{K_1}\right) = 0$$

$$\therefore \quad \frac{c}{K_1} = -\left(1 + \frac{b}{K_{mb}}\right)$$

From definition $K_i' = -c$ = horizontal intercept

$$\therefore \quad K_1 = K_i' \frac{K_{mb}}{K_{mb} + b} \qquad \ldots\ldots\ldots (12B)$$

Now, if $K_b = K_{mb}$ then equation 12A becomes

$$K_c = K_i' \frac{K_{mb}}{K_{mb} + b} \qquad \ldots\ldots\ldots (13A)$$

If, in addition, the K_i' obtained from both the slope and the vertical intercept are equal then equations 12B and 13A are interchangeable.

From equation 7B also K_c can be calculated.

K_i' is obtained by replotting the slope of equation 7B with a fixed - b variable against c. The horizontal intercept then gives K_i' as usual.

$\therefore$ From equation 7B

$$1 + \frac{K_a}{a}\left(1 + \frac{c}{K_c}\right) + \frac{c}{K_1} = 0$$

or $$1 + \frac{K_a}{a} + \frac{K_a c}{a K_c} + \frac{c}{K_1} = 0$$

$$\therefore \quad c\left(\frac{K_a}{a K_c} + \frac{1}{K_1}\right) = -\left(1 + \frac{K_a}{a}\right)$$

Now $K_i' = -c =$ horizontal intercept

$$\therefore \quad K_i' = \frac{1 + \frac{K_a}{a}}{\frac{K_a}{a K_c} + \frac{1}{K_1}}$$

$$\therefore \quad K_i' = \frac{a + K_a}{\frac{K_a}{K_c} + \frac{a}{K_1}} \qquad \ldots\ldots\ldots (14)$$

Equation 14 then gives another equation for calculating K_c from the values of the other constants in equation 14.

If now $K_a = K_{ma}$ and $K_1 = K_c$

then equation 14 reduces to

$$K_i' = \frac{a + K_{ma}}{\frac{K_{ma}}{K_c} + \frac{a}{K_c}} = \frac{a + K_{ma}}{\frac{a + K_{ma}}{K_c}}$$

$$\therefore \quad K_c = K_i' \qquad \ldots\ldots\ldots (15)$$

Equations 10A - 15 therefore enable the true inhibition constants to be calculated from the various apparent inhibition constants obtained from experimental data.

CHAPTER 7

KINETICS OF PHENOL SULPHOTRANSFERASE

Introduction

As mentioned earlier, a specific phenol sulphotransferase devoid of arylamine and steroid sulphotransferase activities and free from PAPS degrading enzymes was obtained by chromatography on DEAE-Sephadex of a partially purified sulphotransferase mixture from guinea pig liver. This enzyme catalyzes the transfer of the sulphuryl group from PAPS to the appropriate phenolic acceptor. In this way the reaction is similar to the transfer, catalyzed by phosphotransferases, of the phosphate group from adenosine 5'-triphosphate (ATP) to the various acceptors. As has been noted earlier three such phosphotransferases have been shown to react with a rapid equilibrium random bi bi mechanism (Reynard, Hass, Jacobsen & Boyer, 1961; Fromm & Zewe, 1962; Morrison & James, 1965). It was of interest therefore to see if the sulphotransferases exhibited similar reaction mechanisms and phenol sulphotransferase was chosen as the model simply because it was obtained as the purest of all the sulphotransferases and was free from any PAPS-degrading enzyme.

In the present study the detailed kinetic investigations were carried out using p-nitrophenol as acceptor and the reaction catalyzed is

$$\underset{\text{(p-nitrophenol)}}{\text{NP}} + \text{PAPS} \rightleftharpoons \underset{\text{(p-nitrophenyl sulphate)}}{\text{NPS}} + \text{PAP}$$

The reaction is readily reversible.

Gregory & Lipmann (1957) and Brunngraber (1958) have used a partially purified phenol sulphotransferase from rabbit liver and have shown that if the reverse reaction is carried out in the presence of another phenol whose sulphate has a lower sulphate group potential than that of NPS, then the sulphate group of NPS can be transferred to the other phenol PAP acting as coenzyme.

Experimental

The preparation of PAPS, the purification of the enzyme and the measurement of sulphotransferase activity have been described earlier. The enzyme was stable for prolonged periods in the cold when kept at pH 7.5 in 0.03M EDTA and 0.01M mercaptoethanol. The enzyme did not require Mg^{2+} ions for its activity and EDTA had no effect. The pH optimum was found to be 5.6 in sodium acetate-acetic acid buffer as shown in Fig. 9.

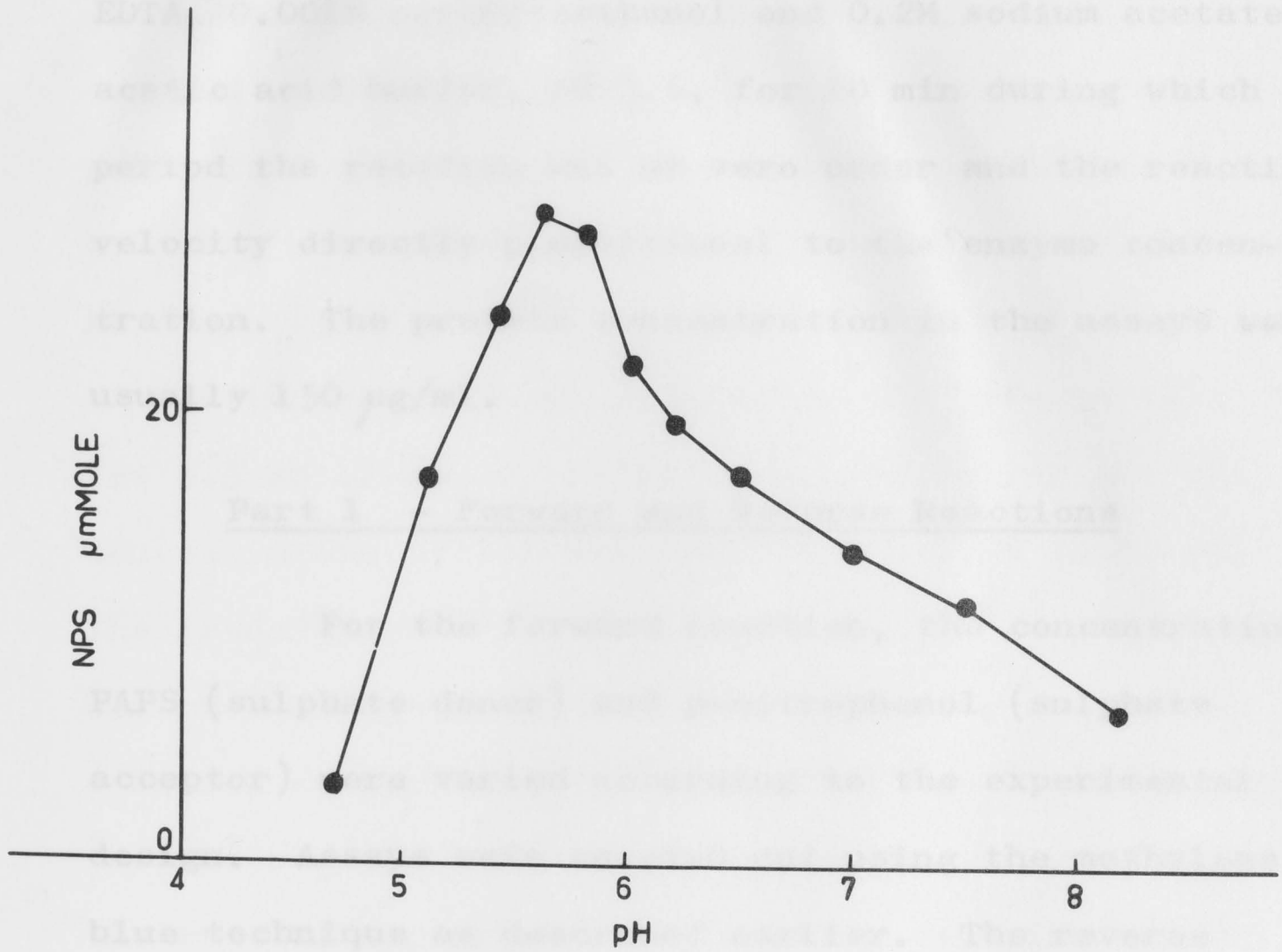


FIG. 9. pH-activity curve of phenol sulphotransferase based on the amount of p-nitrophenyl sulphate (NPS) formed per 10 min.

The general conditions for the enzyme assays were incubation in an aqueous medium containing 0.003M EDTA, 0.001M mercaptoethanol and 0.2M sodium acetate-acetic acid buffer, pH 5.6, for 10 min during which period the reaction was of zero order and the reaction velocity directly proportional to the enzyme concentration. The protein concentration in the assays was usually 150 µg/ml.

Part 1 - Forward and Reverse Reactions

For the forward reaction, the concentration of PAPS (sulphate donor) and p-nitrophenol (sulphate acceptor) were varied according to the experimental design. Assays were carried out using the methylene blue technique as described earlier. The reverse reactions were carried out at the desired concentrations of PAP and p-nitrophenyl sulphate and the rates were measured from the disappearance of p-nitrophenyl sulphate, again using the methylene blue technique.

Part 2 - Specificity and Transfer Reactions

The specificity of phenol sulphotransferase towards various acceptors was determined by replacing p-nitrophenol with the appropriate acceptors in the reaction mixtures : activities were measured by the

methylene blue method. The ability of p-nitrophenyl sulphate to transfer its sulphuryl group to other phenols in the presence of PAP and phenol sulphotransferase was studied by using the reverse reaction with p-nitrophenyl sulphate and PAP in the presence of appropriate amounts of other phenols which could themselves be sulphurylated as shown by specificity studies. The final concentration of NPS in the reaction medium for the reverse reaction was 0.5 mM which was found to be saturating and the concentration of PAP was varied from 1 μM to 40 μM. The transfer reaction media contained 4 mM phenol or 0.1 mM 2-naphthol in addition, depending on whether transfer to phenol or to 2-naphthol was being studied. The reverse and the transfer reactions were measured by the rate of appearance of NP in the reaction mixture, which was taken as a measure of the rate of disappearance of NPS. The rate of appearance of NP was obtained from the absorbance at 400 mμ (using a 1 cm cell) of the reaction mixtures after making them 0.67 N in NaOH. The readings were taken against a blank containing all the ingredients except PAP.

Results

Part 1 - Forward and Reverse Reactions.

The following symbols are used for the forward

reaction:-

v = initial velocity

V_{NP} = initial velocity (NP saturating)

V_{PAPS} = initial velocity (PAPS saturating)

V_m = maximum initial velocity (both NP and PAPS saturating)

Initial Velocity Study (Forward Reaction). The primary reciprocal plots of $\frac{1}{v}$ against $\frac{1}{NP}$ and $\frac{1}{PAPS}$ at fixed PAPS and fixed NP concentrations respectively yielded families of straight lines intersecting on the abscissa (Figs. 10 & 11). The 95% confidence limits of the K_m for NP and PAPS, as calculated by the method of Wilkinson (1961), were

K_m for NP = 0.070 ± 0.012 mM

K_m for PAPS = 0.036 ± 0.010 mM

The replots of the vertical intercepts, that is $\frac{1}{V_{NP}}$ and $\frac{1}{V_{PAPS}}$ against $\frac{1}{PAPS}$ and $\frac{1}{NP}$ yielded straight lines giving the same V_m in both cases and the K_m for PAPS and NP respectively which were equal to the values given above (Fig. 12).

Product Inhibition Study (Forward Reaction). To clarify

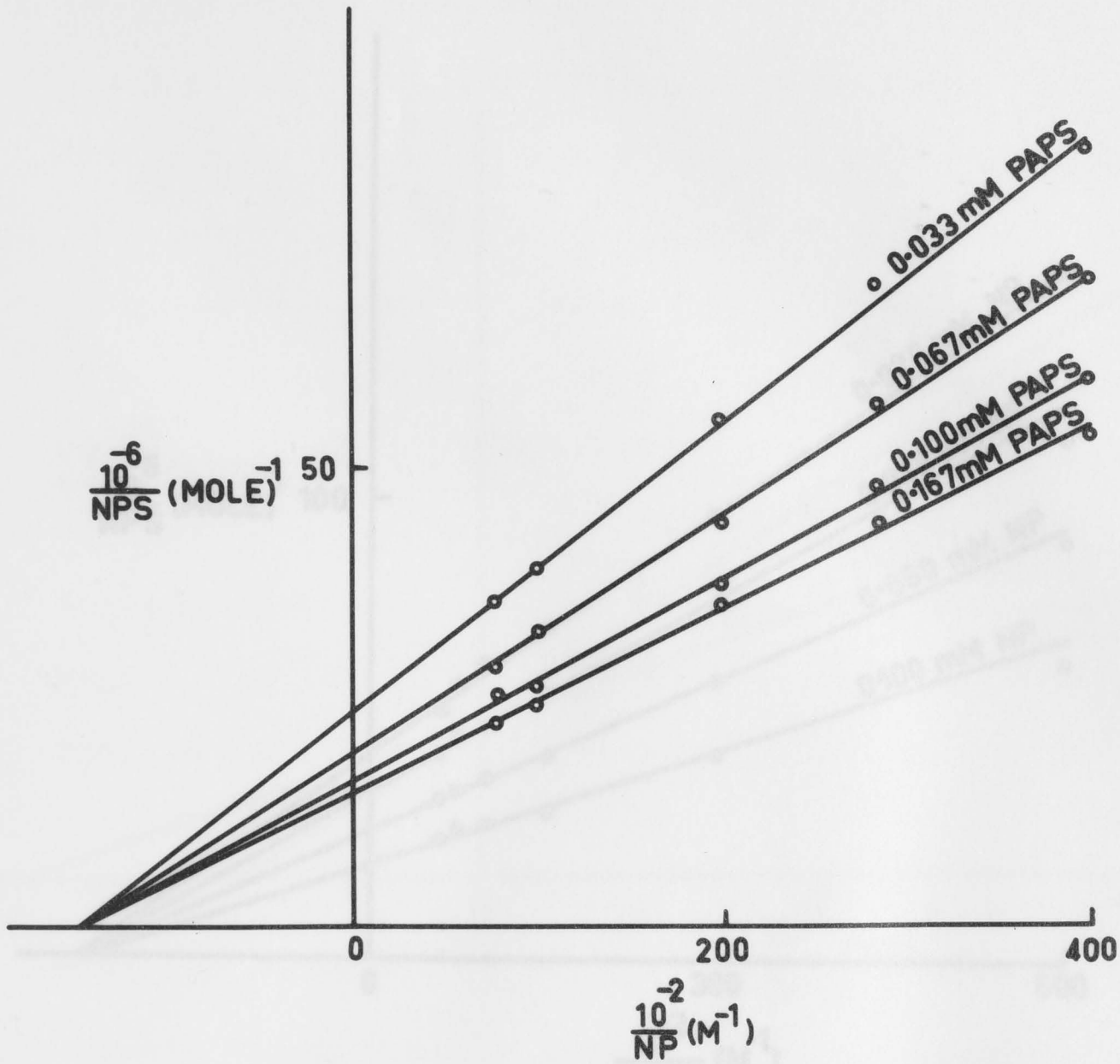


FIG. 10. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of NP at fixed levels of PAPS.

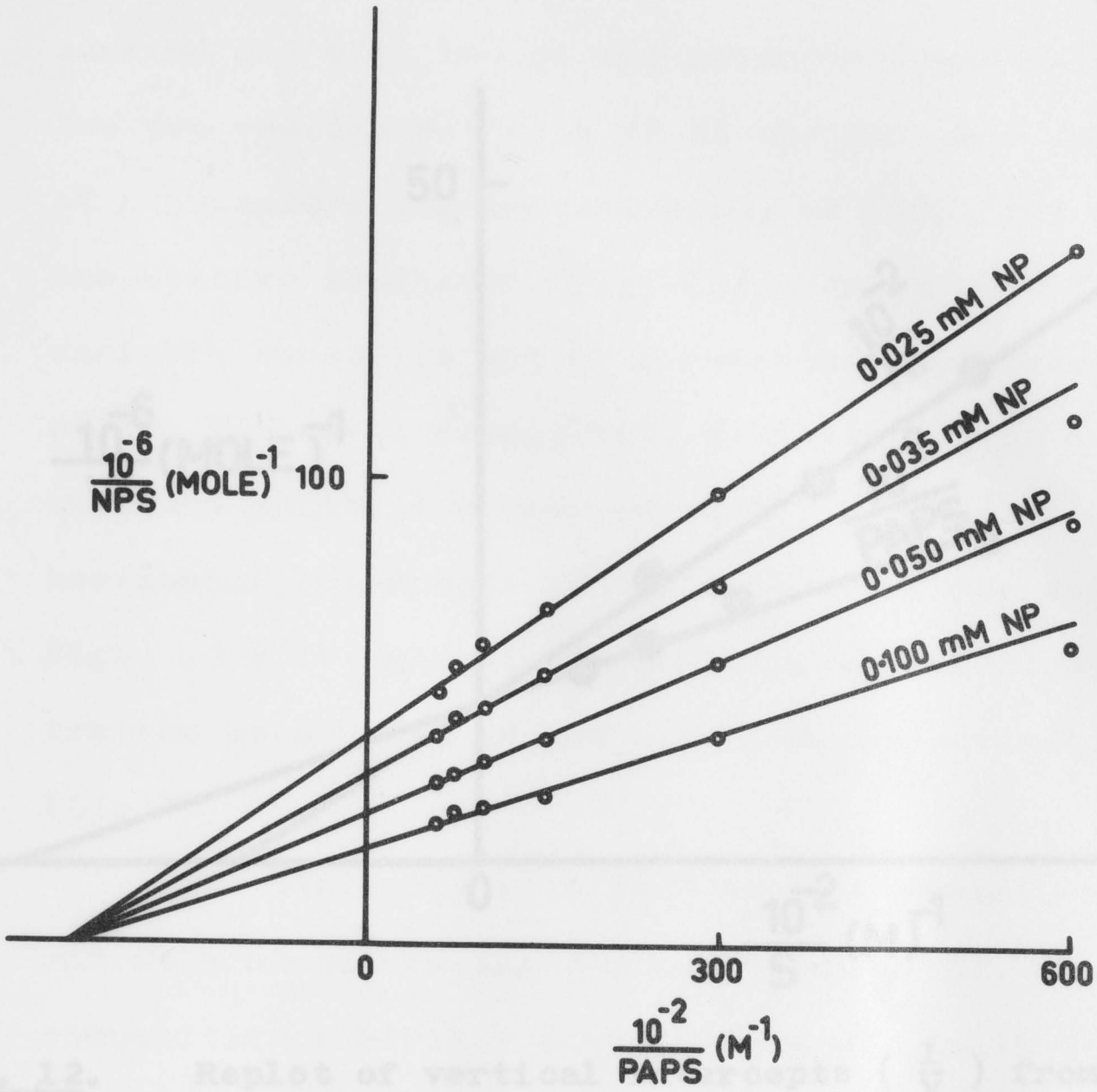


FIG. 11. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of PAPS at fixed levels of NP.

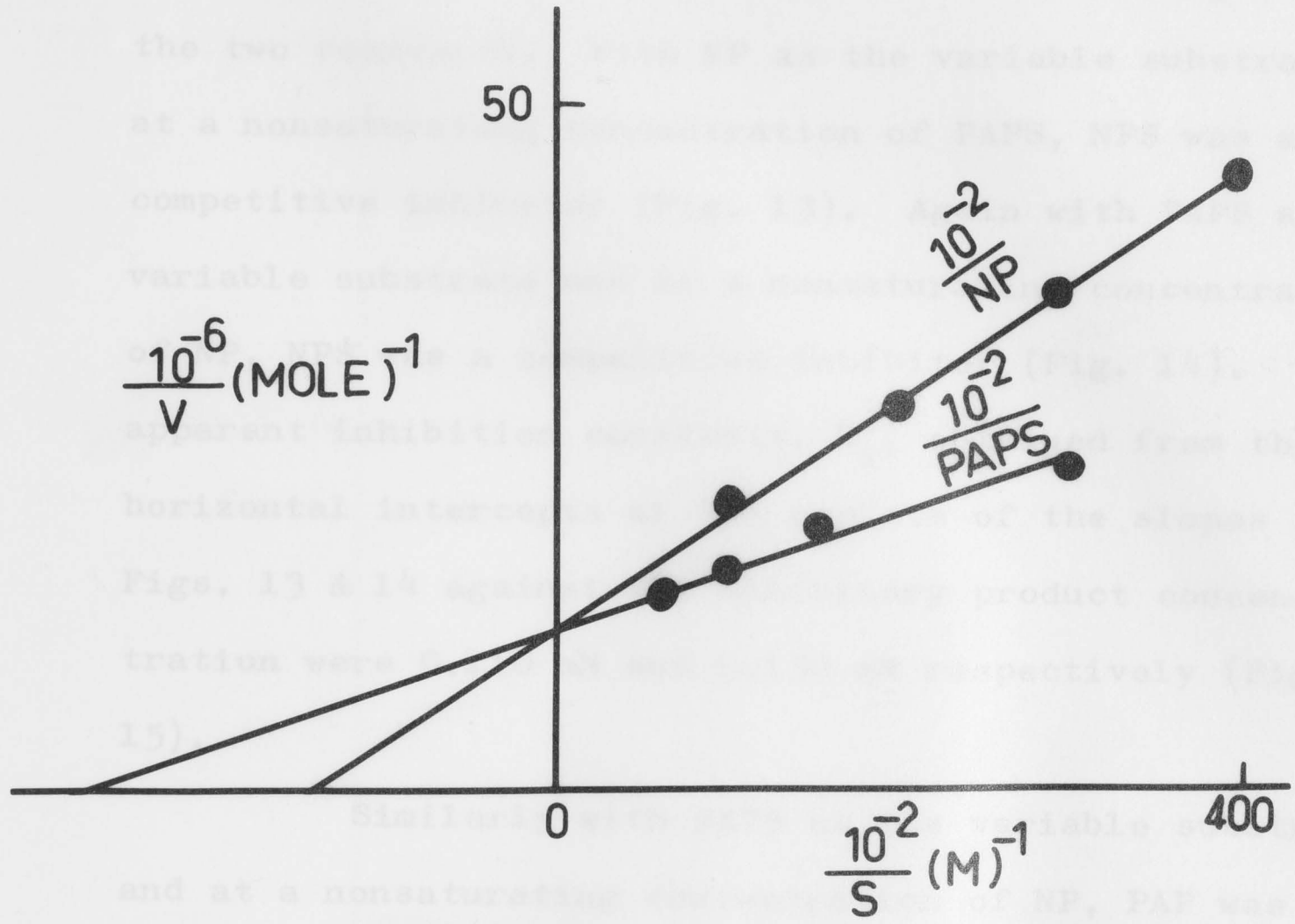


FIG. 12. Replot of vertical intercepts ($\frac{1}{V}$) from Figs. 10 & 11 against reciprocal concentrations ($\frac{1}{S}$) of fixed substrate.

the reaction mechanism product inhibition studies were carried out with one of the products added along with the two reactants. With NP as the variable substrate and at a nonsaturating concentration of PAPS, NPS was a competitive inhibitor (Fig. 13). Again with PAPS as the variable substrate and at a nonsaturating concentration of NP, NPS was a competitive inhibitor (Fig. 14). The apparent inhibition constants, K_i', obtained from the horizontal intercepts of the replots of the slopes from Figs. 13 & 14 against the inhibitory product concentration were 0.110 mM and 0.130 mM respectively (Fig. 15).

Similarly with PAPS as the variable substrate and at a nonsaturating concentration of NP, PAP was a competitive inhibitor with an apparent inhibition constant, K_i', of 0.039 mM (Figs. 16 & 17). On the other hand with NP as the variable substrate and at a nonsaturating level of PAPS, PAP was a noncompetitive inhibitor (Fig. 18). The apparent inhibition constant, K_i', obtained from the slopes was 0.050 mM and that from vertical intercepts was 0.046 mM (Fig. 19).

For convenience the above product inhibition patterns and the K_i' values are summarized in Table 10 as follows:-

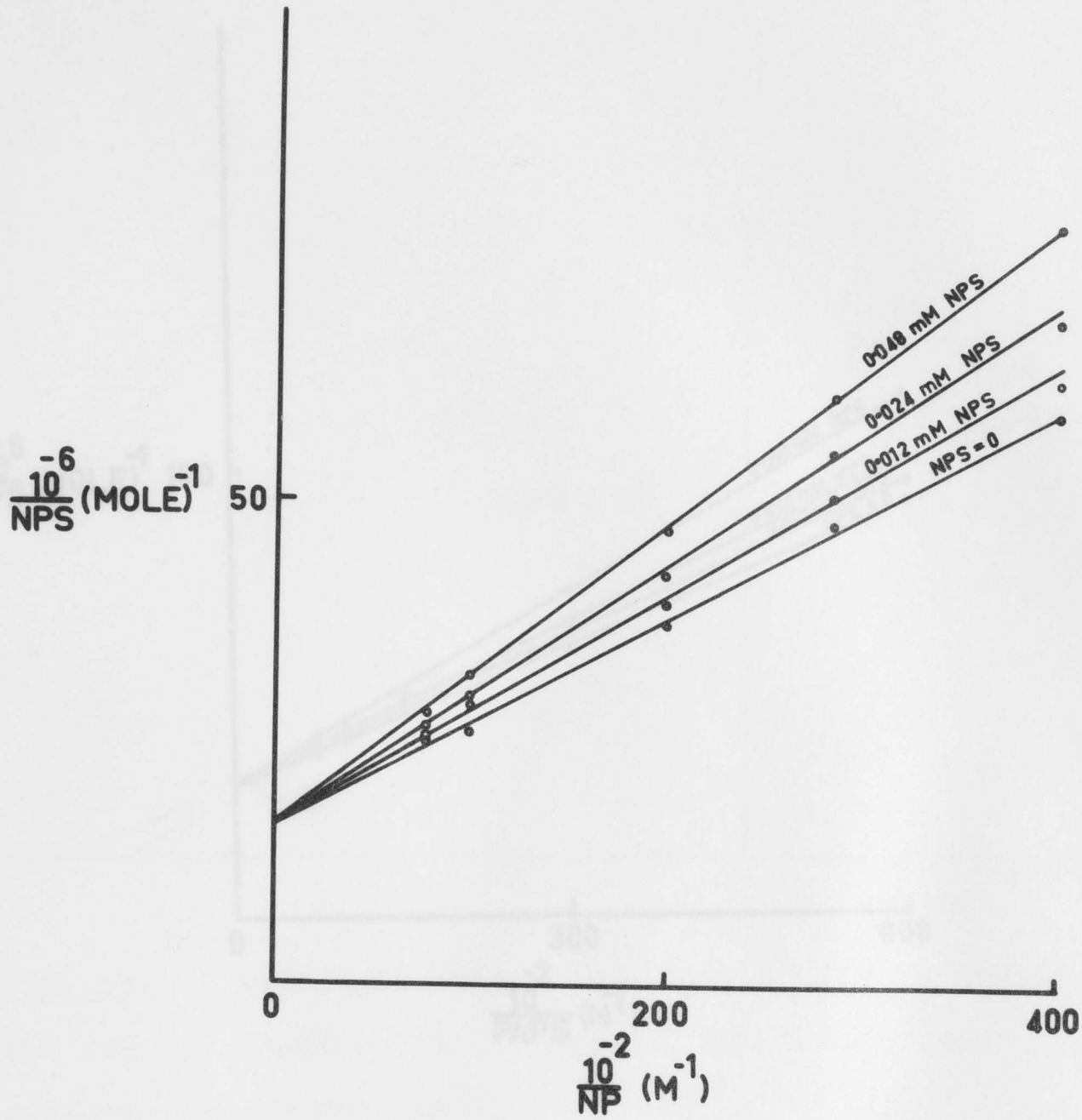


FIG. 13. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of NP at constant PAPS (0.100 mM), showing competitive inhibition by added NPS.

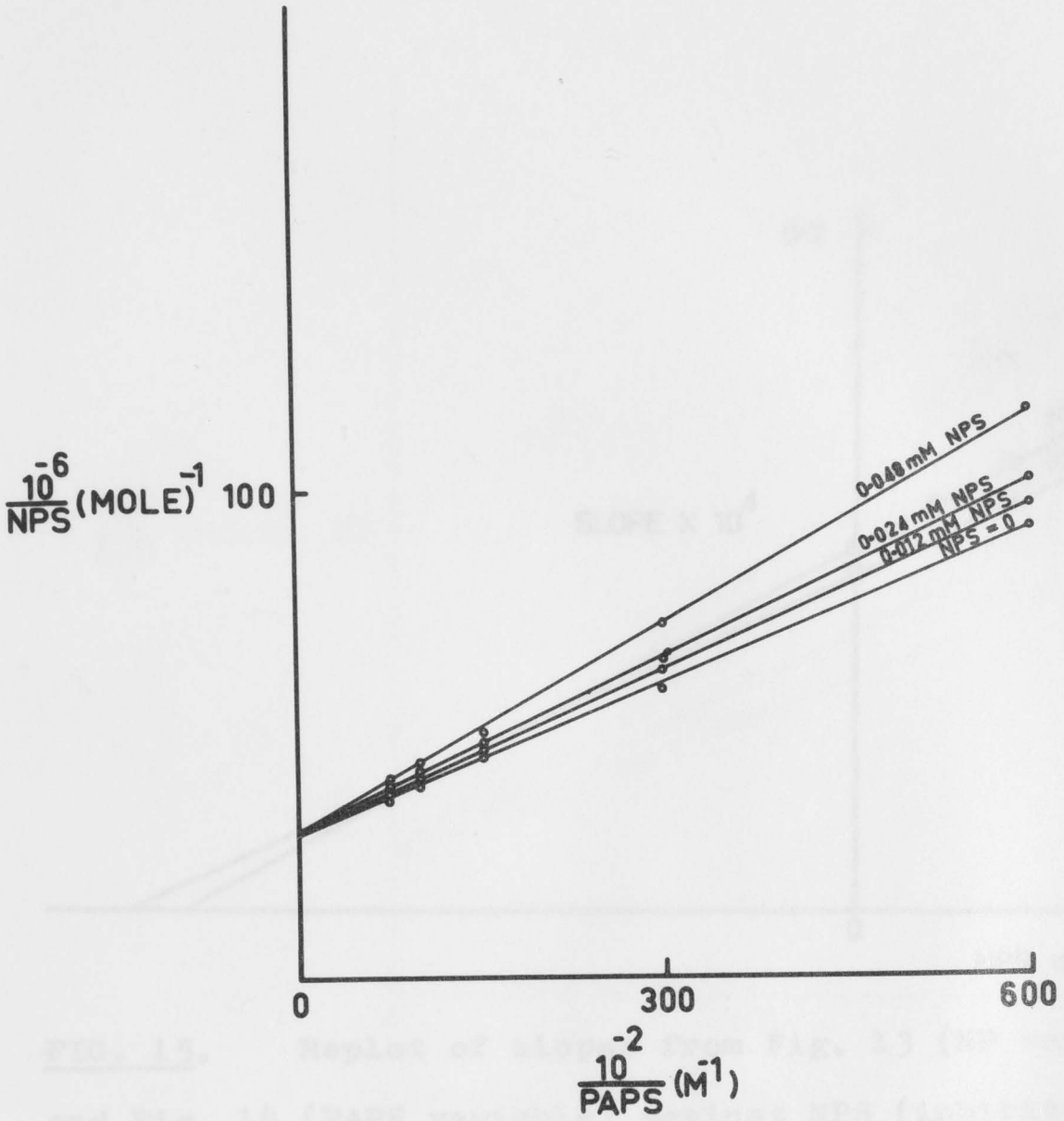


FIG. 14. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of PAPS at constant NP (0.050 mM), showing competitive inhibition by added NPS.

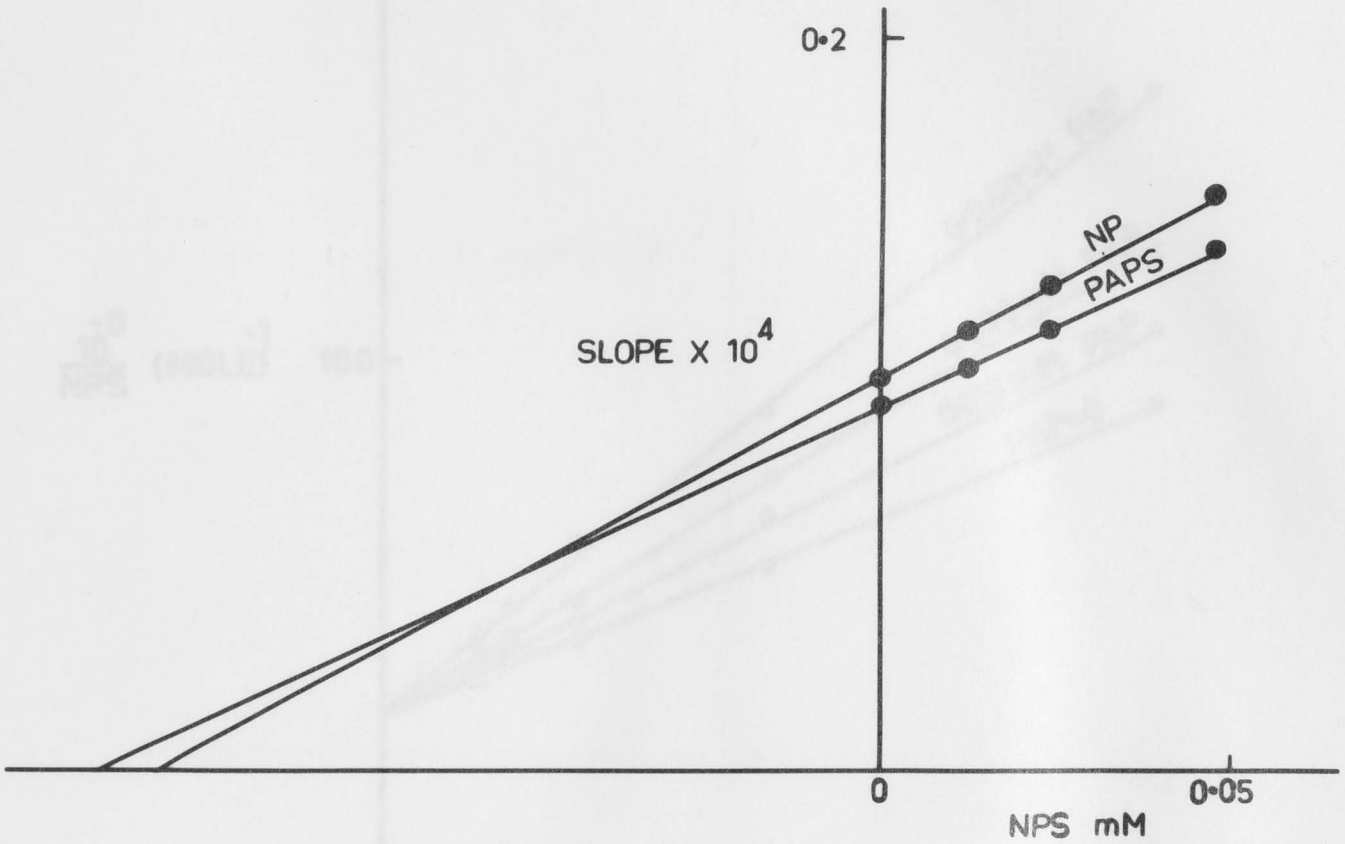


FIG. 15. Replot of slopes from Fig. 13 (NP variable) and Fig. 14 (PAPS variable) against NPS (inhibitory product) concentrations.

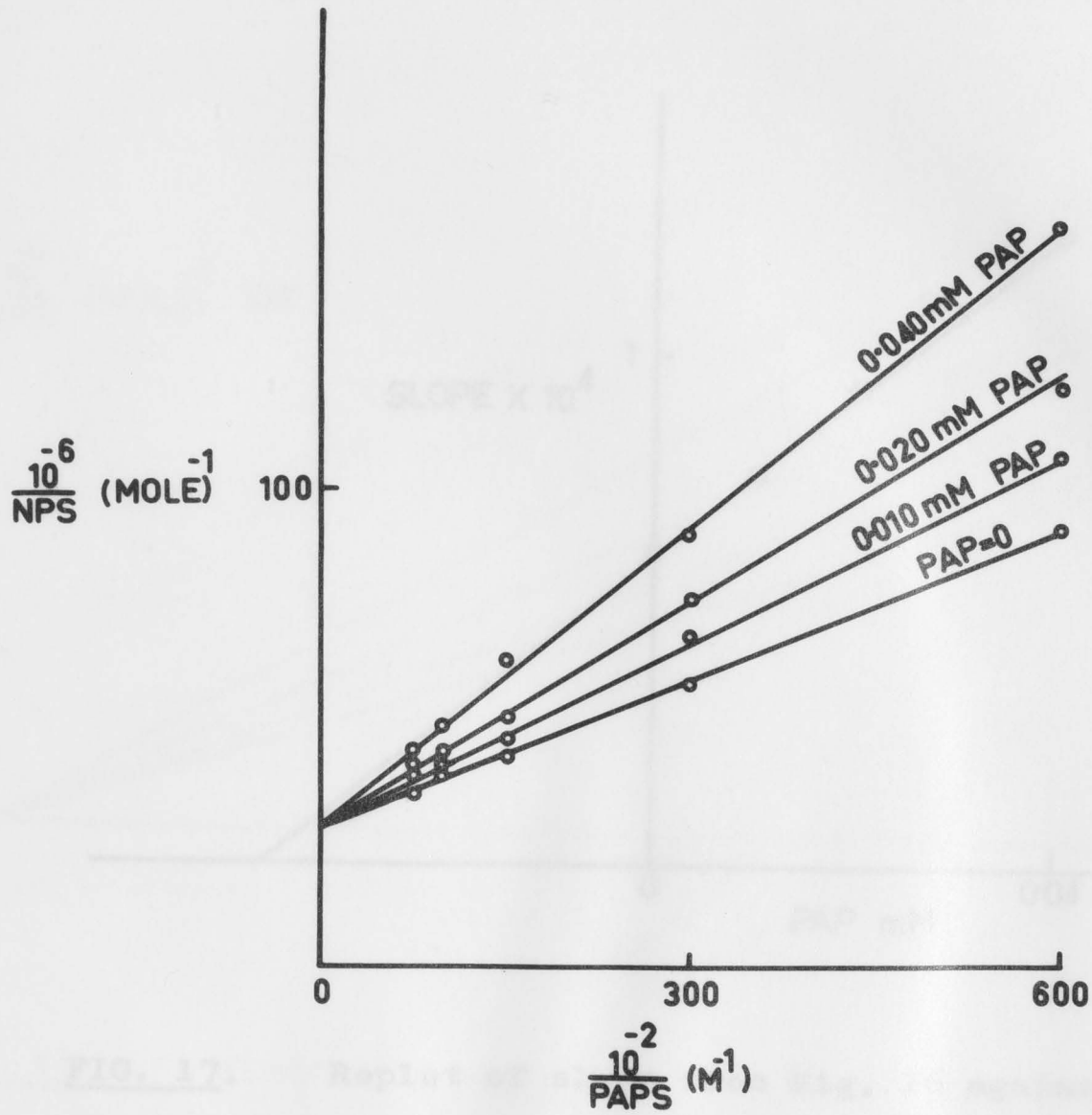


FIG. 16. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of PAPS at constant NP (0.050 mM), showing competitive inhibition by added PAP.

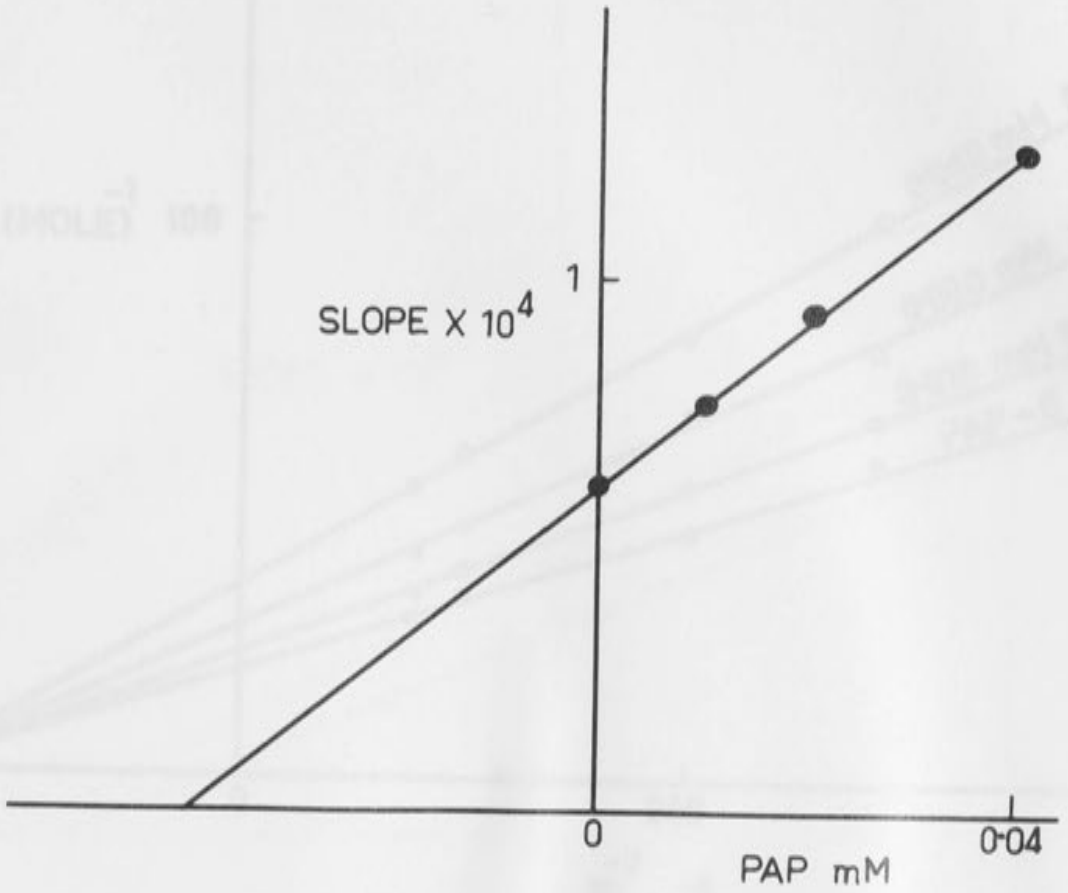


FIG. 17. Replot of slope from Fig. 16 against PAP (inhibitory product) concentrations.

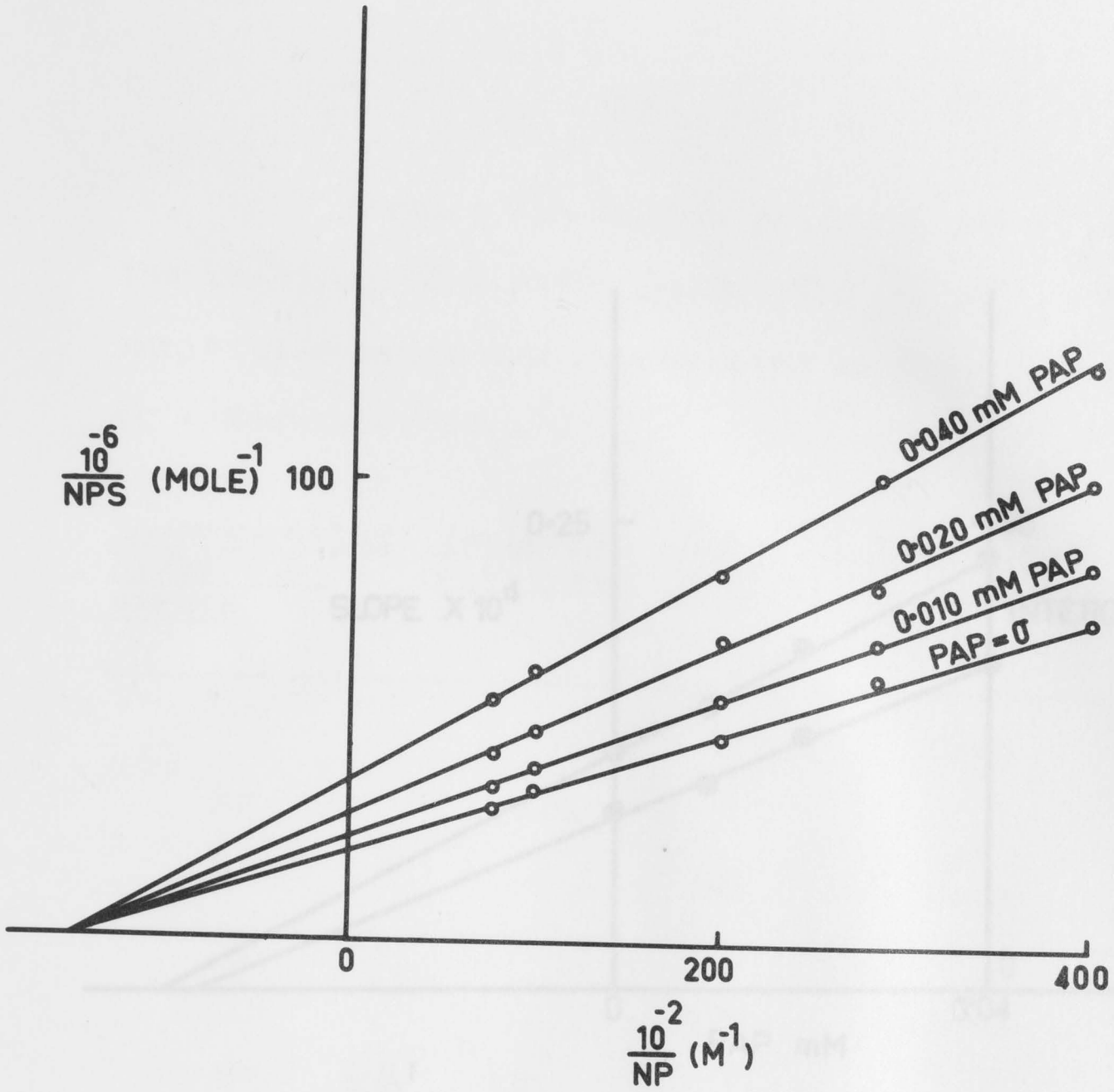


FIG. 18. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of NP at constant PAPS (0.067 mM), showing noncompetitive inhibition by added PAP.

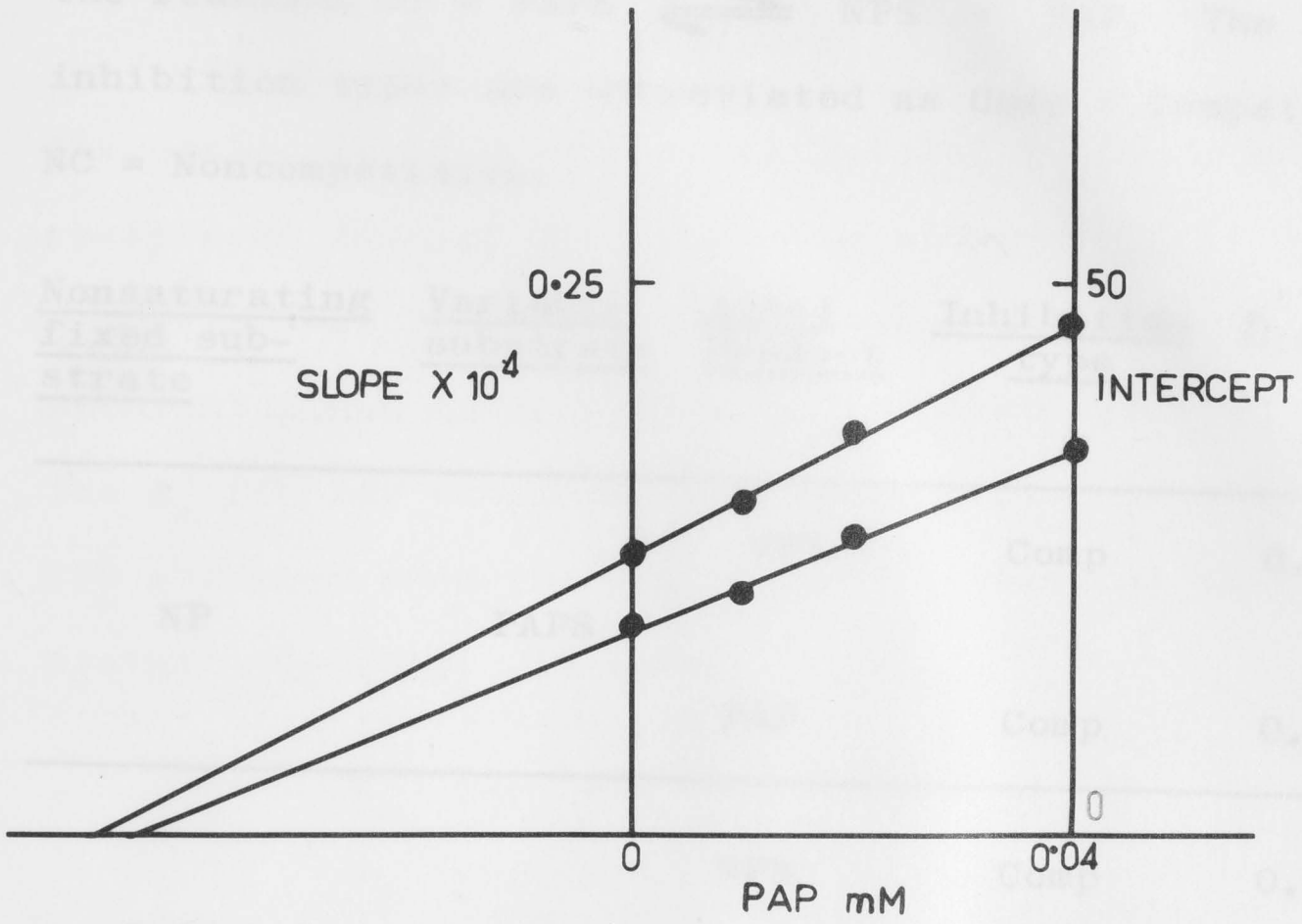


FIG. 19. Replot of slopes and vertical intercepts from Fig. 18 against PAP (inhibitory product) concentrations.

Table 10

Product inhibition patterns and K_i' values for the reaction NP + PAPS $\rightleftharpoons$ NPS + PAP. The inhibition types are abbreviated as Comp = Competitive, NC = Noncompetitive.

Nonsaturating fixed substrate	Variable substrate	Added Product	Inhibition type	K_i', mM
NP	PAPS	NPS	Comp	0.130
		PAP	Comp	0.039
PAPS	NP	NPS	Comp	0.110
		PAP	NC	0.050 (slope)
				0.046 (vertical intercept)

Initial Velocity Study (Reverse Reaction). The reverse reactions were carried out mainly to obtain the V_m' (maximum initial velocity with both NPS and PAP saturating) of the reverse reaction and thereby to calculate the apparent equilibrium constant, K', for the reaction using the appropriate Haldane relationship

(Cleland, 1963a) where

$$K' = \frac{(NPS)(PAP)}{(NP)(PAPS)}$$

The usual plots of reciprocal initial velocity against reciprocal initial concentration of variable substrate, with variable PAP-fixed NPS, yielded a family of straight lines intersecting on the abscissa (Fig. 20). The K_m for PAP obtained from these plots and the K_m for NPS obtained from the replot of the vertical intercepts against $\frac{1}{NPS}$ (Fig. 21) were

$$K_m \text{ for PAP} = 0.014 \text{ mM}$$

$$K_m \text{ for NPS} = 0.110 \text{ mM}$$

The V_m' (maximum initial velocity in the reverse direction) obtained from Fig. 21 and the corresponding V_m (maximum initial velocity in the forward direction) were

$$V_m = 87 \text{ millimicromole/10 min.}$$

$$V_m' = 18 \text{ millimicromole/10 min.}$$

Effect of pH on K_m and V_m (Forward Reaction). The effect of pH on the K_m for both NP and PAPS were studied. The buffers used to cover the pH range were 1M sodium acetate - acetic acid buffer for the pH

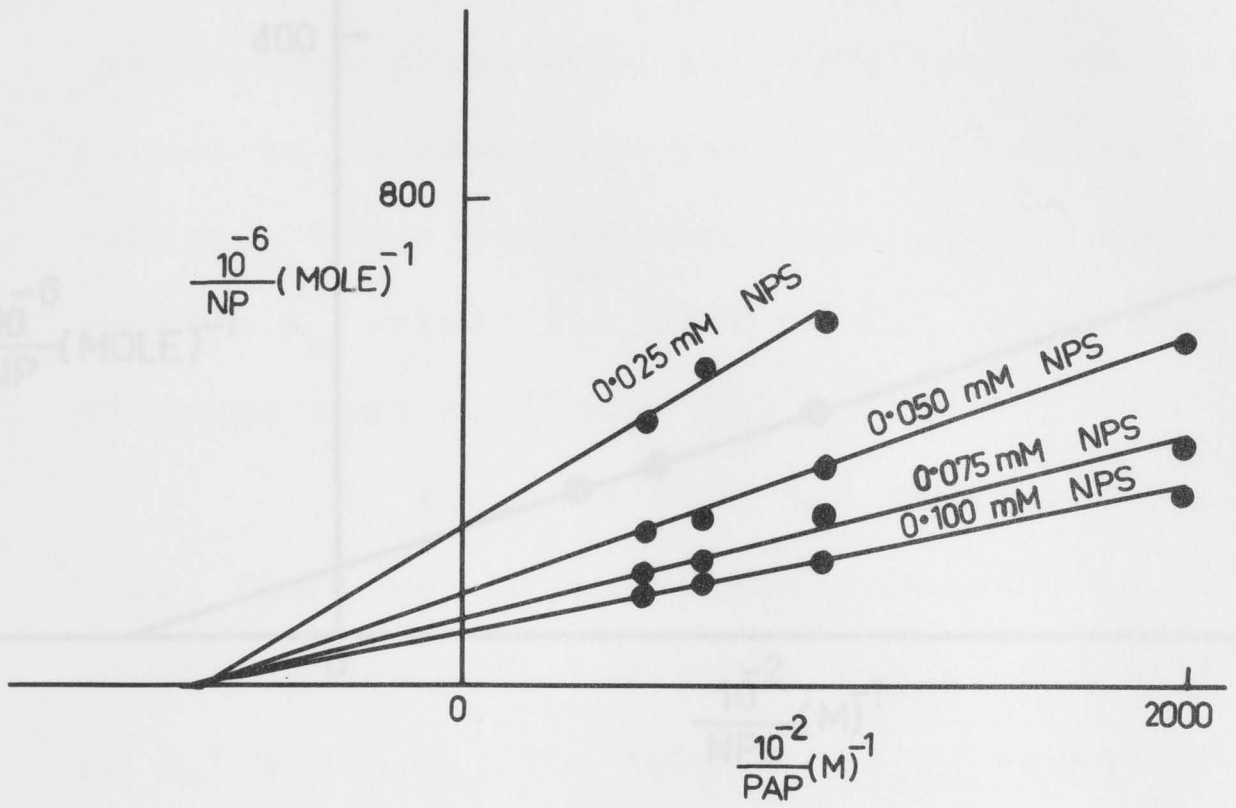


FIG. 20. Plot of reciprocal initial velocity (based on the amount of NP formed per 10 min) against reciprocal concentrations of PAP at fixed levels of NPS.

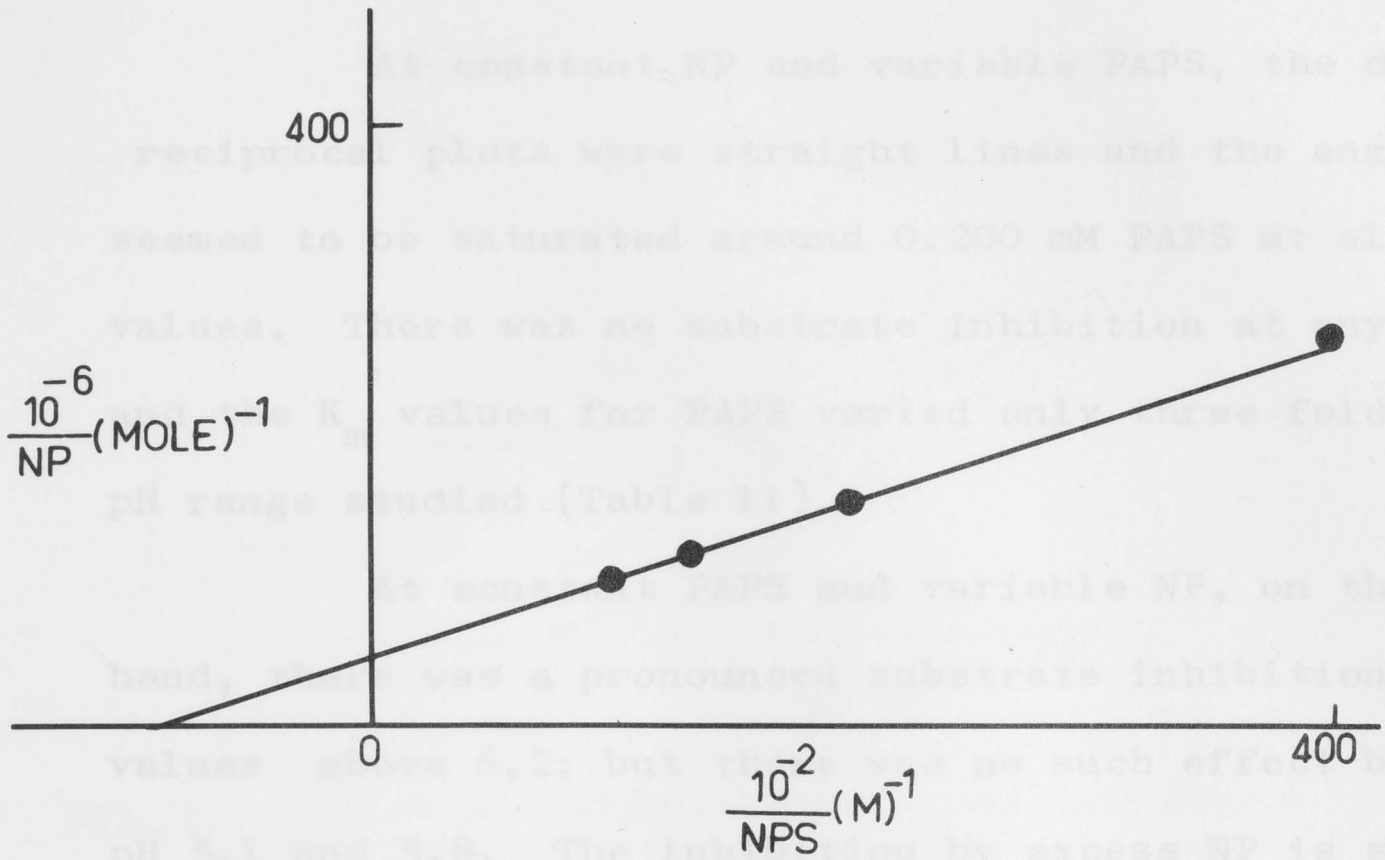


FIG. 21. Replot of vertical intercepts from Fig. 20 against reciprocal concentrations of NPS.

range 5.1 to 5.8, and 1M imidazole-acetic acid buffer for pH range 6.0 to 7.6, the concentration of the buffers in the reaction mixtures being 0.2 M.

At constant NP and variable PAPS, the double reciprocal plots were straight lines and the enzyme seemed to be saturated around 0.200 mM PAPS at all pH values. There was no substrate inhibition at any pH and the K_m values for PAPS varied only three fold in the pH range studied (Table 11).

At constant PAPS and variable NP, on the other hand, there was a pronounced substrate inhibition at pH values above 6.2; but there was no such effect between pH 5.1 and 5.8. The inhibition by excess NP is shown in Fig. 22. For simplicity, reciprocal plots at pH 5.6 and 6.2 have only been shown. It can be seen that although at pH 5.6 the enzyme is saturated at 0.130 mM NP there is no substrate inhibition up to 0.200 mM NP. On the other hand at pH 6.2, substrate inhibition becomes pronounced above 0.100 mM NP and progressively increases at higher pH values. Here again the K_m for NP varied only slightly in the pH range under study (Table 11). The V_m obtained from these plots was also plotted as log V_m against pH (Fig. 23). The figure shows that there are two bends in the curve, one around pH 5.5 and the other at pH 5.9. Since V_m is controlled

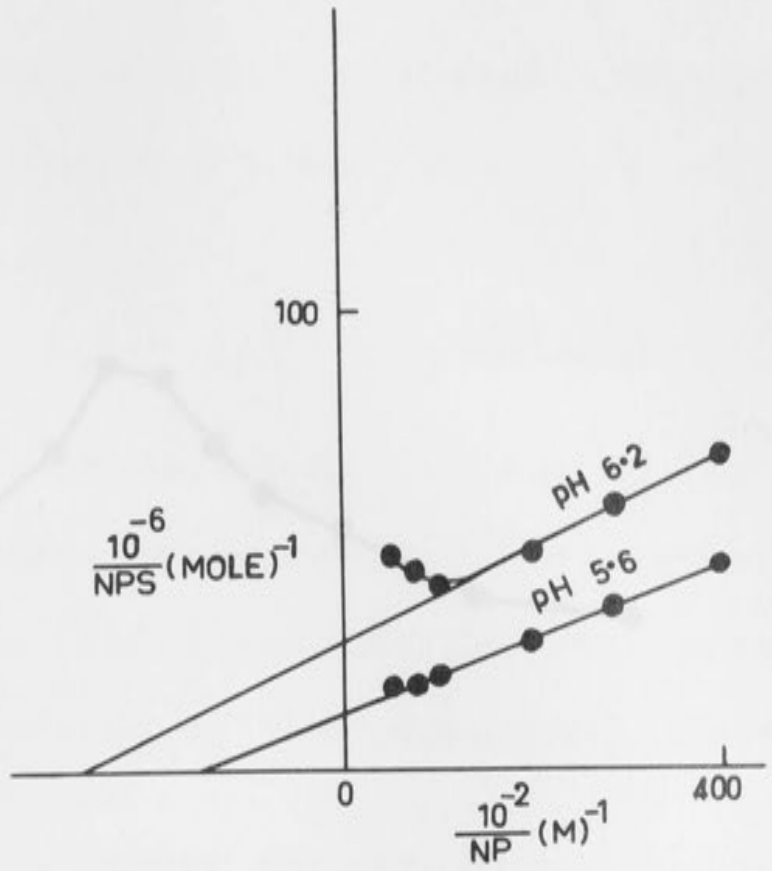


FIG. 22. Plot of reciprocal initial velocity (based on the amount of NPS formed per 10 min) against reciprocal concentrations of NP, showing substrate inhibition at pH 6.2.

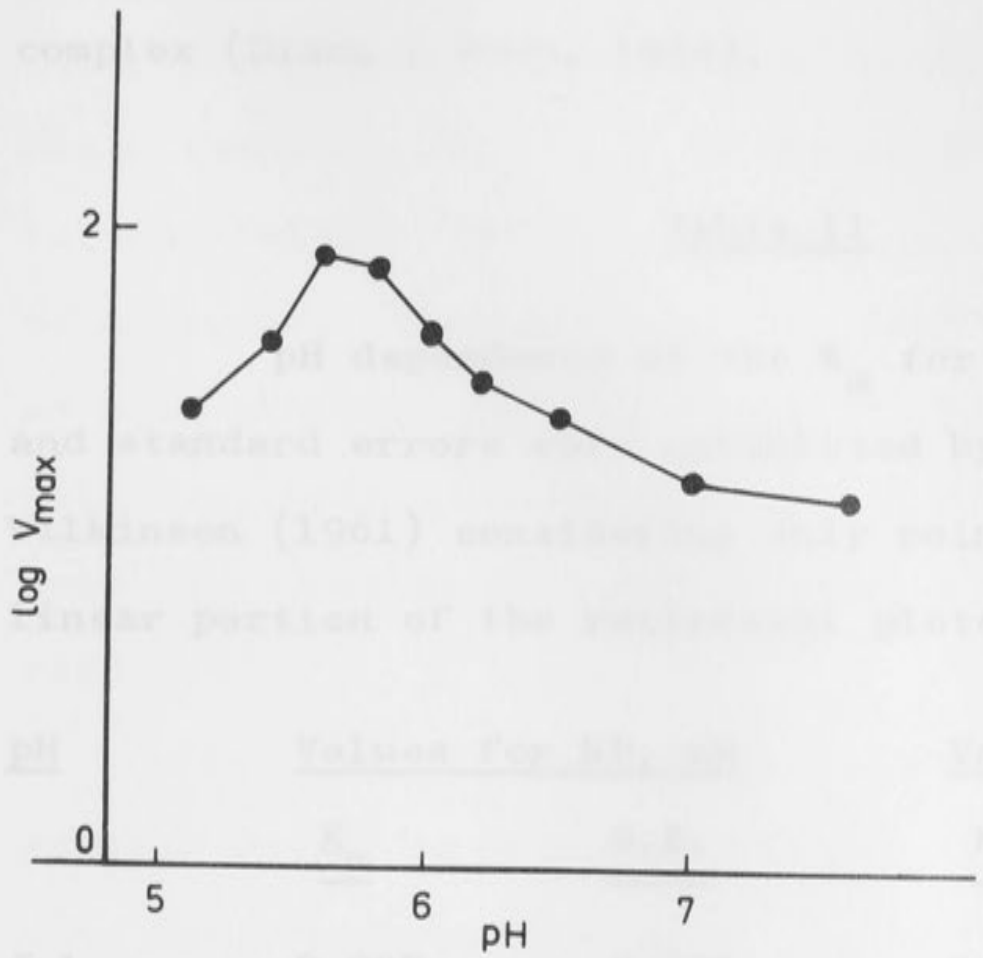


FIG. 23. Plot of log V_m against pH.

by the rate of breakdown of the ternary E.NP.PAPS complex, the two bends probably indicate two pK's of E.NP.PAPS complex (Dixon & Webb, 1964).

Table 11

pH dependence of the K_m for NP and PAPS. K_m and standard errors were calculated by the method of Wilkinson (1961) considering only points forming the linear portion of the reciprocal plots.

pH	Values for NP, mM		Values for PAPS, mM	
	K_m	S.E.	K_m	S.E.
5.1	0.097	0.003	0.038	0.002
5.4	0.069	0.005	0.038	0.002
5.6	0.069	0.002	0.036	0.005
5.8	0.059	0.002	0.039	0.007
6.0	0.033	0.003	0.094	0.008
6.2	0.029	0.002	0.103	0.008
6.5	0.028	0.006	0.072	0.015
7.0	0.024	0.005	0.048	0.003
7.6	0.021	0.002	0.040	0.002

Part 2 - Specificity and Transfer Reactions

Specificity. Initial velocity studies were carried out with variable concentrations of potential acceptors at a fixed level of PAPS to give the plots of reciprocal initial velocity against reciprocal initial concentration of acceptor. Then assuming the same reaction mechanism as when p-nitrophenol is acceptor, V_{max} (maximum initial velocity, both substrates saturating) were calculated from the vertical intercepts using a constant K_m for PAPS.

The initial velocity experiments conducted in greater detail with 2-naphthol (N) confirmed that the same basic mechanism as with p-nitrophenol was obeyed. The results showed that with 2-naphthol as acceptor, the K_m for 2-naphthol was independent of the concentration of PAPS and vice versa (Figs. 24 & 25). The 95% confidence limits for the K_m for 2-naphthol and PAPS as calculated by the method of Wilkinson (1961) were

K_m for 2-naphthol = 0.025 ± 0.004 mM

K_m for PAPS = 0.063 ± 0.017 mM

Application of Student's 't' test showed that the above K_m for PAPS was not significantly different from the K_m for PAPS obtained using p-nitrophenol as

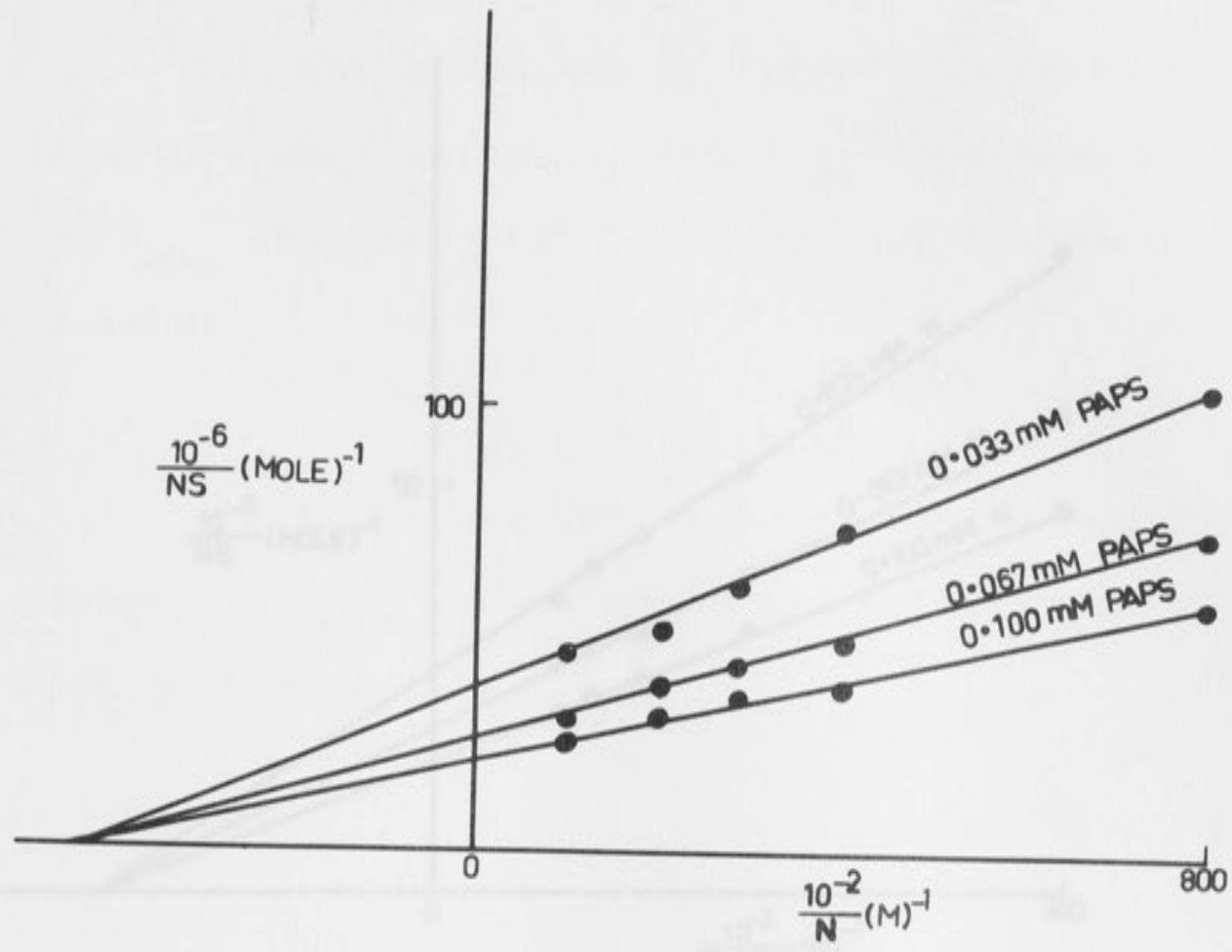


FIG. 24. Plot of reciprocal initial velocity (based on the amount of 2-naphthyl sulphate = NS formed per 10 min) against reciprocal concentrations of 2-naphthol (N) at fixed levels of PAPS.

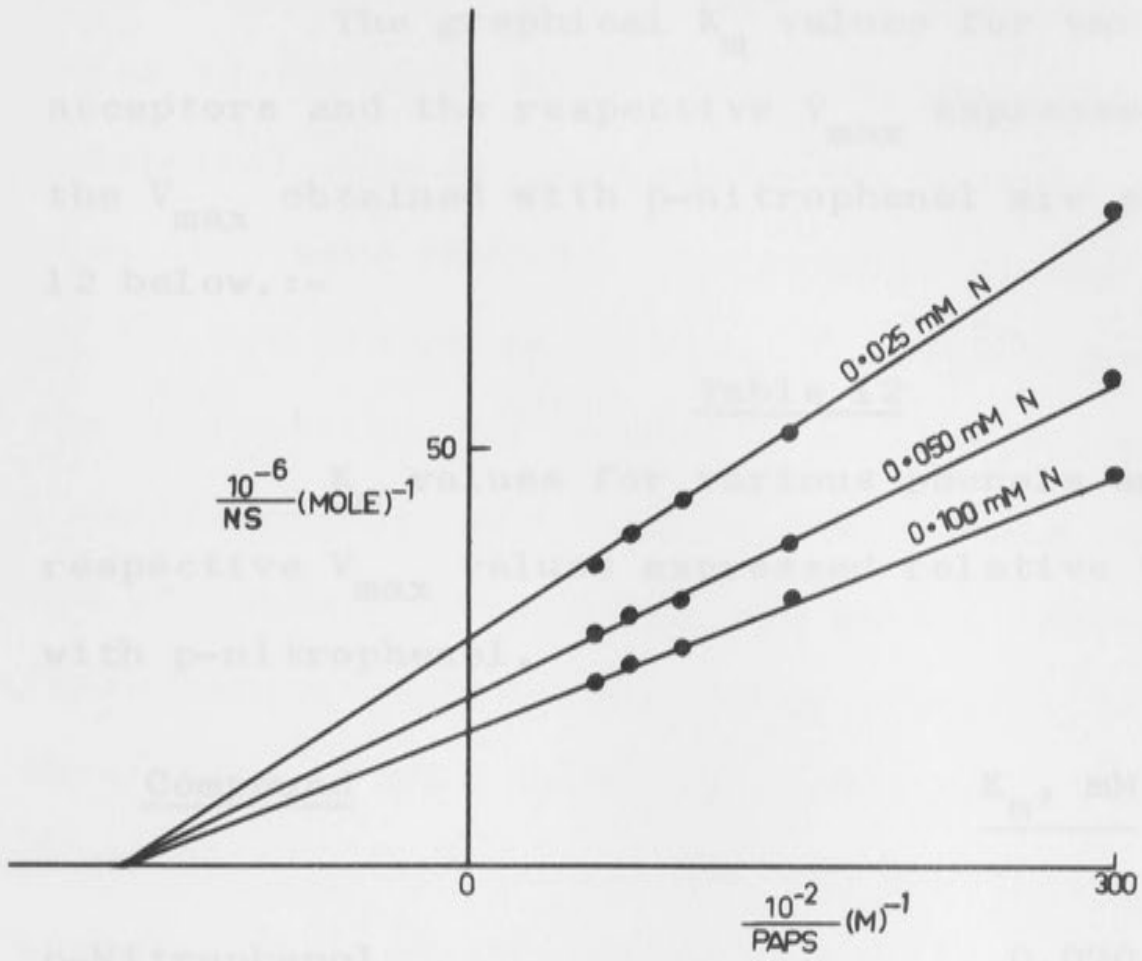


FIG. 25. Plot of reciprocal initial velocity (based on the amount of 2-naphthyl sulphate = NS formed per 10 min) against reciprocal concentrations of PAPS at fixed levels of 2-naphthol (N).

acceptor.

The graphical K_m values for various acceptors and the respective V_{max} expressed relative to the V_{max} obtained with p-nitrophenol are shown in Table 12 below.:-

Table 12

K_m values for various phenols and the respective V_{max} values expressed relative to the V_{max} with p-nitrophenol.

Compound	K_m, mM	Relative V_{max}
p-Nitrophenol	0.070	1.00
Phenol	2.50	0.30
1-Naphthol	0.025	1.02
2-Naphthol	0.025	0.95
4-Nitro-1-naphthol	0.018	0.39
Phenanthrol	0.017	0.51
Hydroxycyclopentenophenanthrene	0.015	0.13
Equilin	0.020	0.21
Equilenin	0.015	0.10

Transfer Reactions. The ability for NPS to transfer its sulphuryl group to phenol and 2-naphthol was investigated by incubating NPS and PAP with the enzyme in the

absence and presence of phenol or 2-naphthol. The rate of the transfer reaction was then assumed to be equal to the rate of NP formation when phenol or 2-naphthol were present minus the rate of the reaction when they were absent. The results of the reactions at pH 5.6 are presented in Fig. 26. The figure shows that the increase in the formation of NP was approximately 1 millimicromole and 0.25 millimicromole per 10 min in the presence of 2-naphthol and phenol respectively. These values corresponded to an increase in the absorbance of only 0.020 and 0.005 per 10 min in the presence of added 2-naphthol and phenol respectively, assuming a molar absorption coefficient of 18×10^3 for NP at 400 mμ. Fig. 26 also shows that there was no substrate inhibition by PAP up to 0.040 mM concentration in the assay. The experiments were repeated with a different preparation of PAP both at the usual pH of 5.6 and also at pH 7.5 using Tris-acetic acid buffer. The results from these experiments were again similar to those of Fig. 26 and no significant amount of transfer could be detected. Gregory & Lipmann (1957) who studied the transfer reactions at pH 7.8 using a phenol sulphotransferase from rabbit liver obtained a change of absorbance at 400 mμ of 0.05 per min even at as low a concentration of PAP as 0.8 μM.

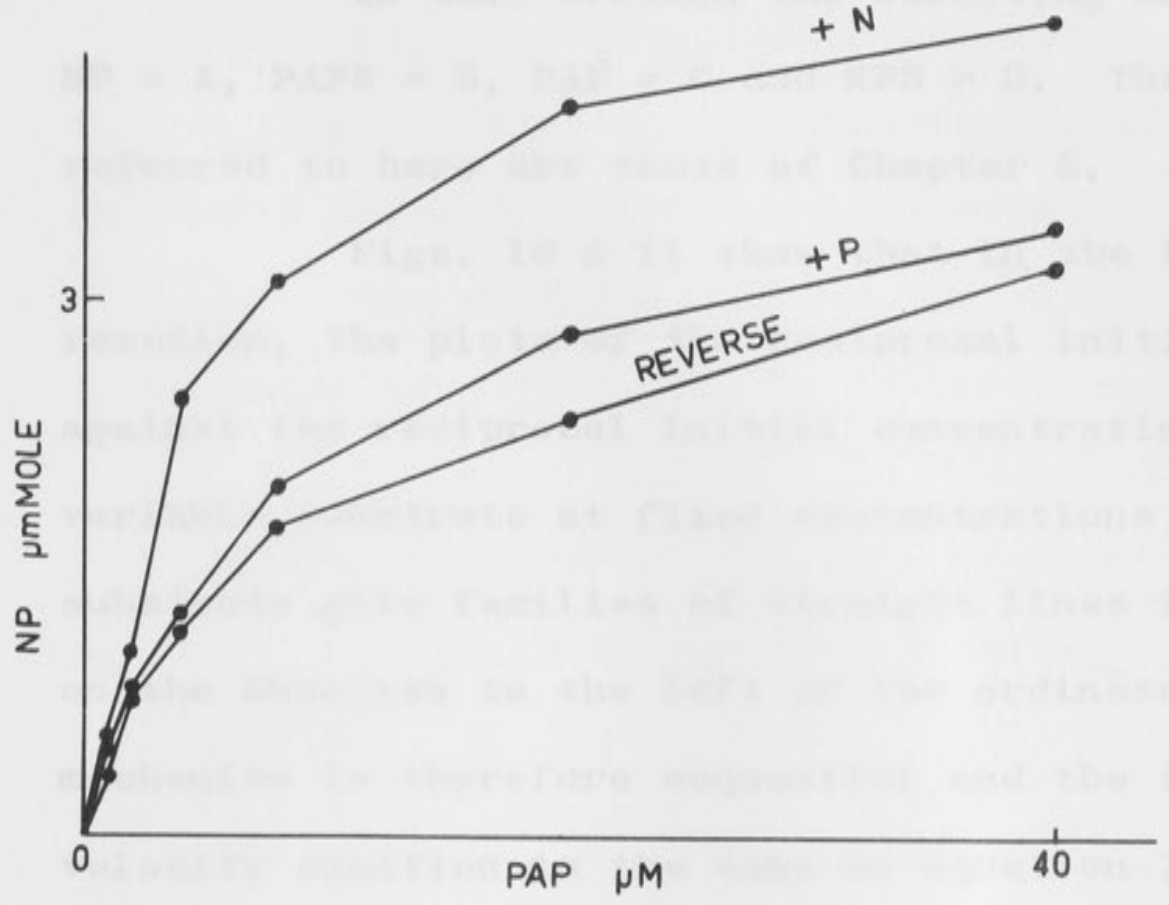


FIG. 26. Amount of NP formed per 10 min (at varying concentrations of PAP and a constant concentration of NPS of 0.5 mM) by the reverse reaction and the transfer reactions in the presence of 4 mM phenol (+P) and 0.1 mM 2-naphthol (+N).

Discussion

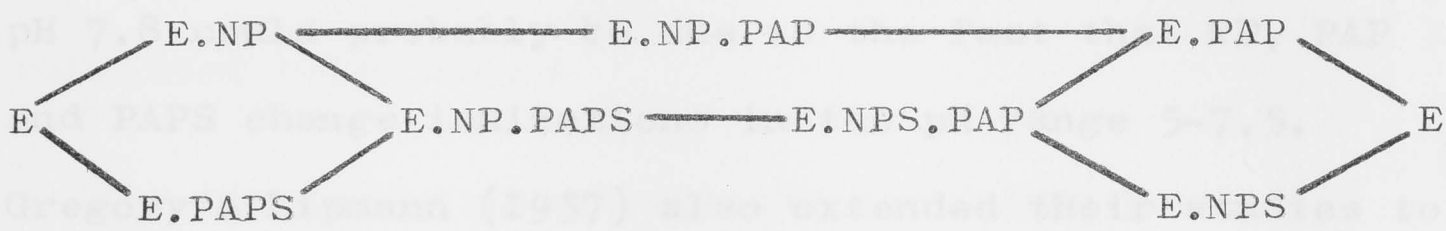
In this section the following are assumed: NP = A, PAPS = B, PAP = C and NPS = D. The equations referred to here are those of Chapter 6.

Figs. 10 & 11 show that in the forward reaction, the plots of the reciprocal initial velocities against the reciprocal initial concentrations of the variable substrate at fixed concentrations of the other substrate give families of straight lines intersecting on the abscissa to the left of the ordinate. The mechanism is therefore sequential and the initial velocity equation is the same as equation 2. Hence $K_a = K_{ma}$ and $K_b = K_{mb}$. The initial velocity studies therefore limit the reaction mechanism to two types, namely the rapid equilibrium random mechanism and the ordered mechanism. The product inhibition patterns shown in Figs. 13-19 and summarized in Table 10 are incompatible with compulsory pathways and are consistent only with a rapid equilibrium random mechanism in which a dead end ternary complex, EAC, that is E.NP.PAP, is formed (Table 9). The other possible dead end complex EBD (E.NPS.PAPS) does not form probably due to steric hindrance. The product inhibition by NPS can then be represented by equations 6A and 6B and

the inhibition by PAP by equations 9A and 9B.

The initial velocity studies in the reverse direction (Fig. 20) also indicated that $K_c = K_{mc}$ and $K_d = K_{md}$, where K_{mc} and K_{md} are the limiting Michaelis constants for PAP and NPS respectively.

The full reaction mechanism can be represented as



All the steps except the interconversion of E.NP.PAPS to E.NPS.PAP adjust rapidly.

The apparent equilibrium constant, K', for the reaction was then obtained by the use of the Haldane relationship for the rapid equilibrium random mechanism (Cleland, 1963a) which in this particular case reduces to

$$K' = \frac{V_m K_{mc} K_{md}}{V'_m K_{ma} K_{mb}}$$

where $K' = \frac{(NPS)(PAP)}{(NP)(PAPS)}$

The value for K' obtained from the Haldane relationship

was 3.0. The only other value for this equilibrium constant is that of Gregory & Lipmann (1957) who used a partially purified phenol sulphotransferase from rabbit liver to establish the equilibrium of the reaction in 2-3 hours at pH 7.8. From the amount of the components remaining at equilibrium they calculated a K' of 26 for the reaction involving p-nitrophenol. The difference in the two K' values, one at pH 5.6 and the other at pH 7.8 could probably be due to the fact that NP, PAP and PAPS change ionizations in the pH range 5-7.5. Gregory & Lipmann (1957) also extended their studies to the reaction involving 3,5-dinitrophenol whose sulphate also has a high sulphate group potential and obtained a value of 4.1 for K'. The apparent equilibrium constant obtained by the Haldane relationship above will therefore seem to be of the same order of magnitude as the values obtained by Gregory & Lipmann (1957) for the reactions involving p-nitrophenol and 3,5-dinitrophenol both of which form sulphates of high sulphate group potential so that the reverse reactions are appreciable.

The apparent inhibition constants, K_i', obtained from product inhibition experiments were calculated from the statistical K_m and V values (Wilkinson, 1961) at different inhibitor levels. The true inhibition constants, namely K_d and K_c were then

obtained by applying equations (11A, 11B) and (13A,15) respectively. The standard errors (S.E.) of the calculated K_d and K_c were obtained from the standard deviation, S, of the values by applying the formula

$$S.E. = \frac{S}{n^{\frac{1}{2}}}$$

where n = number of observations.

The mean values of K_d and K_c obtained from different experiments and their standard errors are tabulated below where

K_d = Dissociation constant for E.NPS complex

K_c = Dissociation constant for E.PAP complex

For comparison, the K_m for NPS and that for PAP have also been given.

	Mean	S.E.
K_d (from variable PAPS expt.), mM	0.068	0.008
K_d (from variable NP expt.), mM	0.034	0.012
K_{md} (K_m for NPS), mM	0.110	
K_c (from variable PAPS expt.), mM	0.024	0.002
K_c (from variable NP expt.), mM	0.019	0.001
K_{mc} (K_m for PAP), mM	0.014	

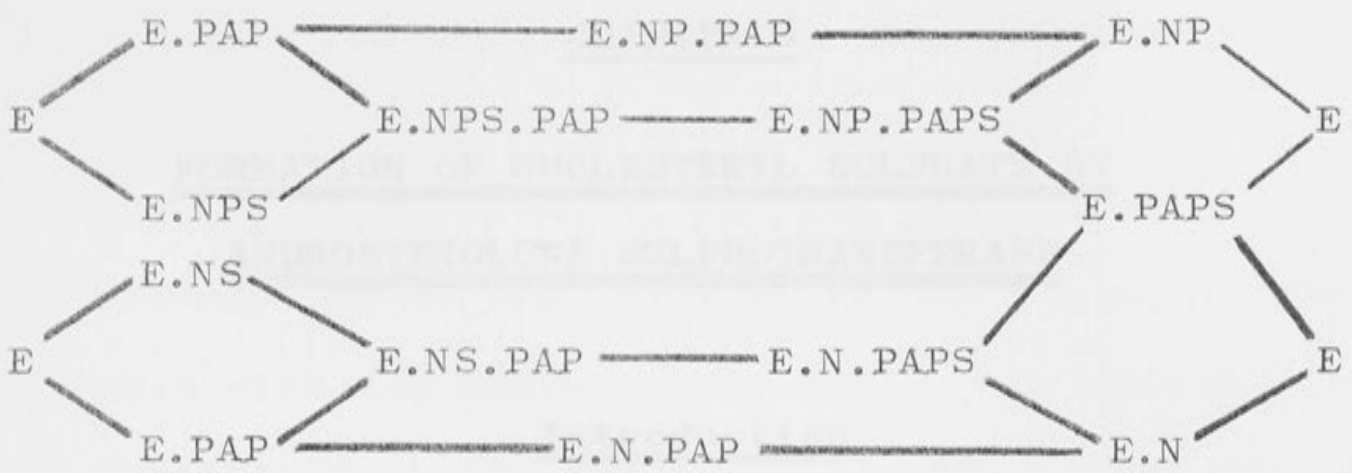
The above results show that the values for K_d and K_c

obtained from different experiments and calculated on the basis of rapid equilibrium random mechanism with one dead end EAC complex are essentially equal and they approximate K_{md} and K_{mc} values respectively taking into account the possible experimental errors. These results therefore give further evidence in support of the mechanism suggested for the reaction. The precision of the experiments with NPS(D) as added product is lower than when PAP(C) is the added product, because the former experiments are subject to greater experimental errors as NPS added to the reaction mixture had to be kept low to work in the sensitive range of the spectrophotometer.

From the specificity studies it is clear that phenol sulphotransferase catalyzes the sulphuryl-ation of all the phenols so far tested with the exception of oestrone. The latter differs structurally from the other phenols in that the phenolic ring is adjacent to a rigid and nonplanar cyclohexane ring. It is therefore suggested that a phenol may only be a substrate for phenol sulphotransferase if it is a simple benzene derivative or, in the case of polycyclic phenols, if the ring adjacent to the phenolic ring is either aromatic or quinonoid in nature.

The transfer of the sulphuryl group of NPS to 2-naphthol or phenol in the presence of PAP and

phenol sulphotransferase from guinea pig liver was not significant. In this way the results differ from those of Gregory & Lipmann (1957) and of Brunngraber (1958) who used phenol sulphotransferase from rabbit liver and found rapid rates of transfer of the sulphuryl group from NPS to phenol and m-aminophenol respectively. The enzyme under consideration here must therefore have very different kinetic properties from that of rabbit liver. It is however difficult to see why no significant transfer reaction was detected in the present instance, especially in the case of 2-naphthol, because it can be seen in Table 12 that the enzyme exhibits the same V_{max} with both 2-naphthol and p-nitrophenol and that the K_m for 2-naphthol and p-nitrophenol are similar. A quantitative interpretation of the transfer reaction can be given only when the mechanism for transfer is solved. In the light of the mechanism given above for the sulphurylation of p-nitrophenol, the transfer reaction can be envisaged as follows, where N = 2-naphthol and NS = 2-naphthyl sulphate.



A dead end ternary complex E.N.PAP in analogy with E.NP.PAP has been assumed.

CHAPTER 8

FORMATION OF CHOLESTERYL SULPHATE BY ANDROSTENOLONE SULPHOTRANSFERASE

Introduction

From the partial separation of the steroid sulphotransferases described in an earlier chapter it has been possible to show for the first time that androstenolone sulphotransferase will catalyze the transfer of the sulphuryl group from PAPS to cholesterol forming cholesteryl sulphate, and in the light of the present thinking that this ester may be a direct precursor of the hormonal steroids, it is of interest to study its formation by androstenolone sulphotransferase. Although cholesteryl sulphate has been isolated from blood (Drayer & Lieberman, 1965), adrenal gland (Drayer, Roberts, Bandi & Lieberman, 1964) and brain (Moser, Moser & Orr, 1966), all previous attempts to demonstrate its synthesis in vitro had failed (Schneider & Lewbart, 1956; Nose & Lipmann, 1958; Baulieu et al., 1965).

It has been suggested earlier that the apparent arylamine sulphotransferase activity of guinea pig liver is due in part to the activity of androsteno-

lone sulphotransferase. As the former activity is inhibited by androstenolone methyl ether (which cannot itself be sulphurylated), and the inhibition is apparently due to the reaction of the D ring of the steroid with the enzyme (Roy, 1961), the effect of this 17-oxosteroid on the synthesis of cholesteryl sulphate is of obvious interest. Further, if cholesteryl sulphate is an intermediary in the conversion of cholesterol to androstenolone sulphate and thence to androstenolone (Roberts et al., 1964a,b) or to oestrogens (Baulieu, 1962) then the effect of this 17-oxosteroid on the sulphurylation of cholesterol would be all the more important.

Experimental

Androstenolone sulphotransferse was prepared exactly as described earlier. The reaction mixture had a volume of 1 ml and contained 0.1M Tris-acetic acid buffer, pH 7.5, 0.001M mercaptoethanol and 0.005M magnesium acetate in excess of the amount of EDTA added with the enzyme preparation, together with the appropriate concentrations of PAPS and of cholesterol, the latter being added in 0.1 ml of propylene glycol. After incubation at 37^{o} for 30 min the reaction was

stopped by adding 5 ml of ethanol. The cholesteryl sulphate formed was then determined exactly as described earlier. The effect of androstenolone methyl ether on the reaction was studied by adding the appropriate amount from a solution of this steroid in propylene glycol and adjusting the concentration of cholesterol solution so that the concentration of propylene glycol was maintained at 10% in the reaction mixture.

Results

The reciprocal plots of the initial velocity data obtained from variable cholesterol-fixed PAPS and variable PAPS-fixed cholesterol experiments are presented in Figs. 27 & 28. Thus the K_m for cholesterol was not altered by changes in the concentration of PAPS, nor was the K_m for PAPS affected by changes in the concentration of cholesterol.

With regard to the effect of androstenolone methyl ether on the reaction, preliminary results showed that at equimolar concentrations of cholesterol and androstenolone methyl ether, the initial velocity was inhibited by about 50%. More detailed studies of the inhibition using different inhibitor concentrations showed that androstenolone methyl ether was a competitive

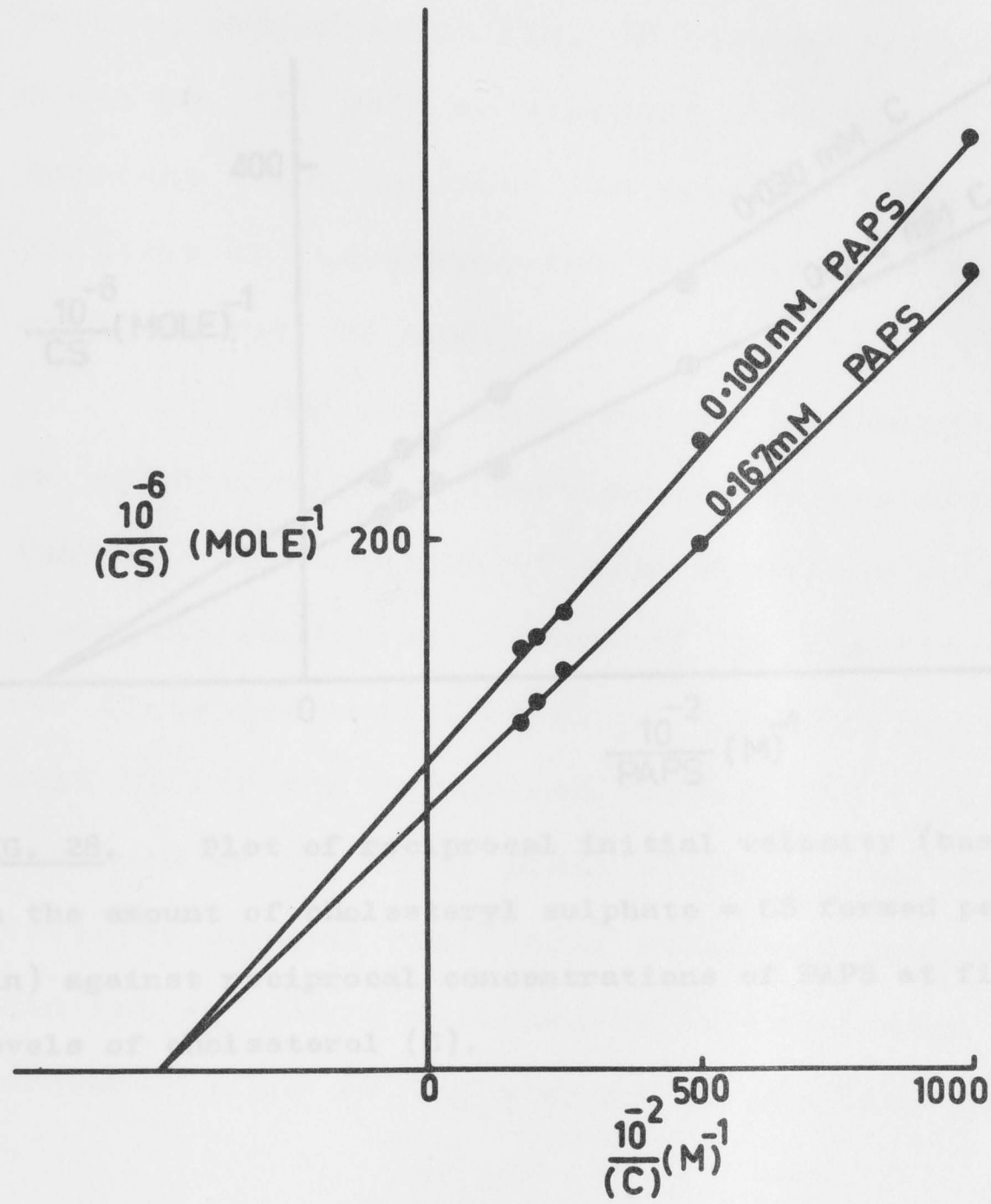


FIG. 27. Plot of reciprocal initial velocity (based on the amount of cholesteryl sulphate = CS formed per 30 min) against reciprocal concentrations of cholesterol (C) at fixed levels of PAPS.

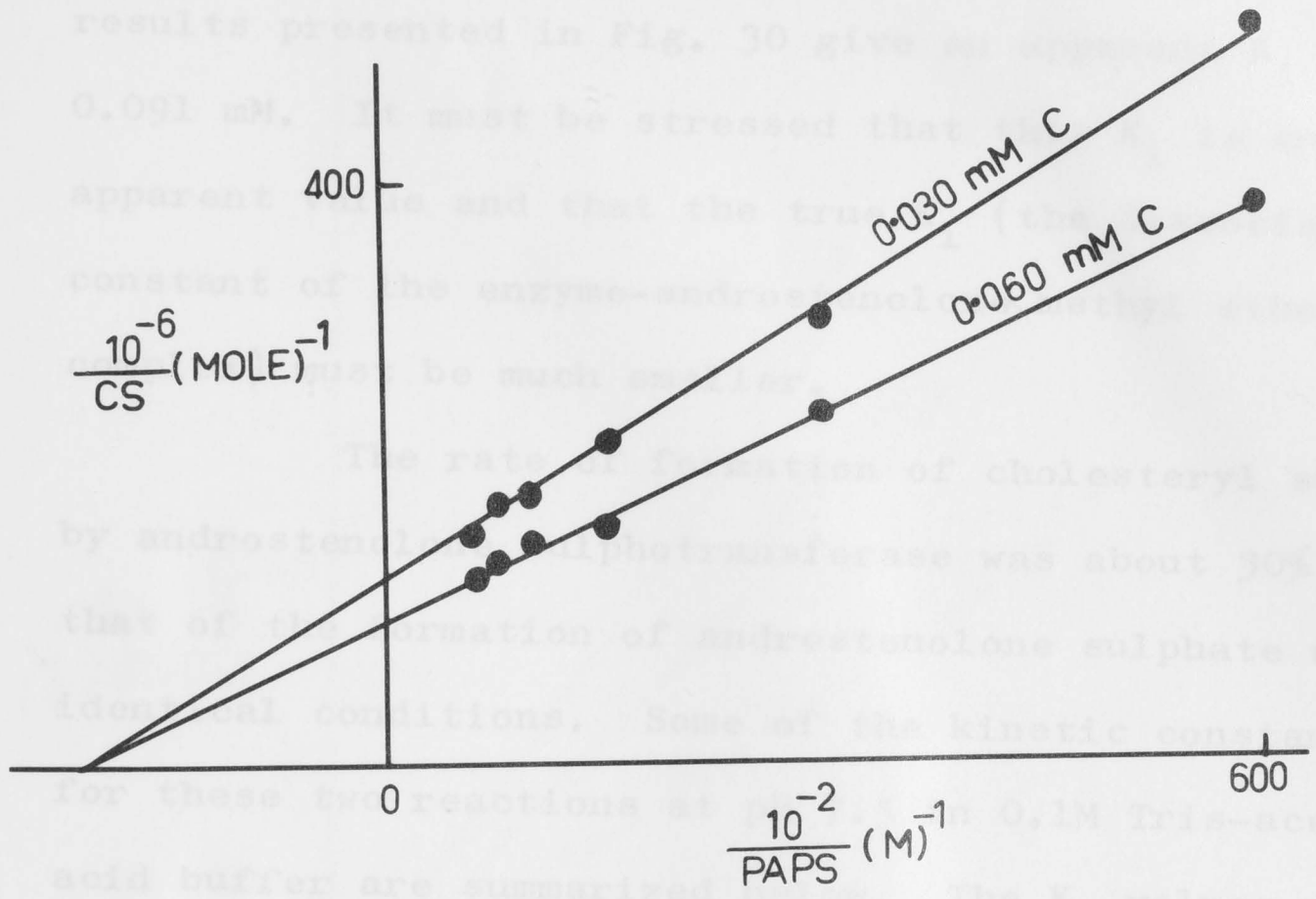


FIG. 28. Plot of reciprocal initial velocity (based on the amount of cholesteryl sulphate = CS formed per 30 min) against reciprocal concentrations of PAPS at fixed levels of cholesterol (C).

inhibitor with respect to cholesterol at a fixed nonsaturating concentration of PAPS (Fig. 29). The results presented in Fig. 30 give an apparent K_i of 0.091 mM. It must be stressed that this K_i is an apparent value and that the true K_i (the dissociation constant of the enzyme-androstenolone methyl ether complex) must be much smaller.

The rate of formation of cholesteryl sulphate by androstenolone sulphotransferase was about 30% of that of the formation of androstenolone sulphate under identical conditions. Some of the kinetic constants for these two reactions at pH 7.5 in 0.1M Tris-acetic acid buffer are summarized below. The K_m values were obtained by the usual plots of reciprocal initial velocity against reciprocal substrate concentrations and the V_{max} were obtained by extrapolation to infinite concentrations of the steroid in question and of PAPS.

	Androstenolone	Cholesterol
K_m for steroid (mM)	0.020	0.020
K_m for PAPS (mM)	0.043	0.050
Relative V_{max}	1.00	0.38

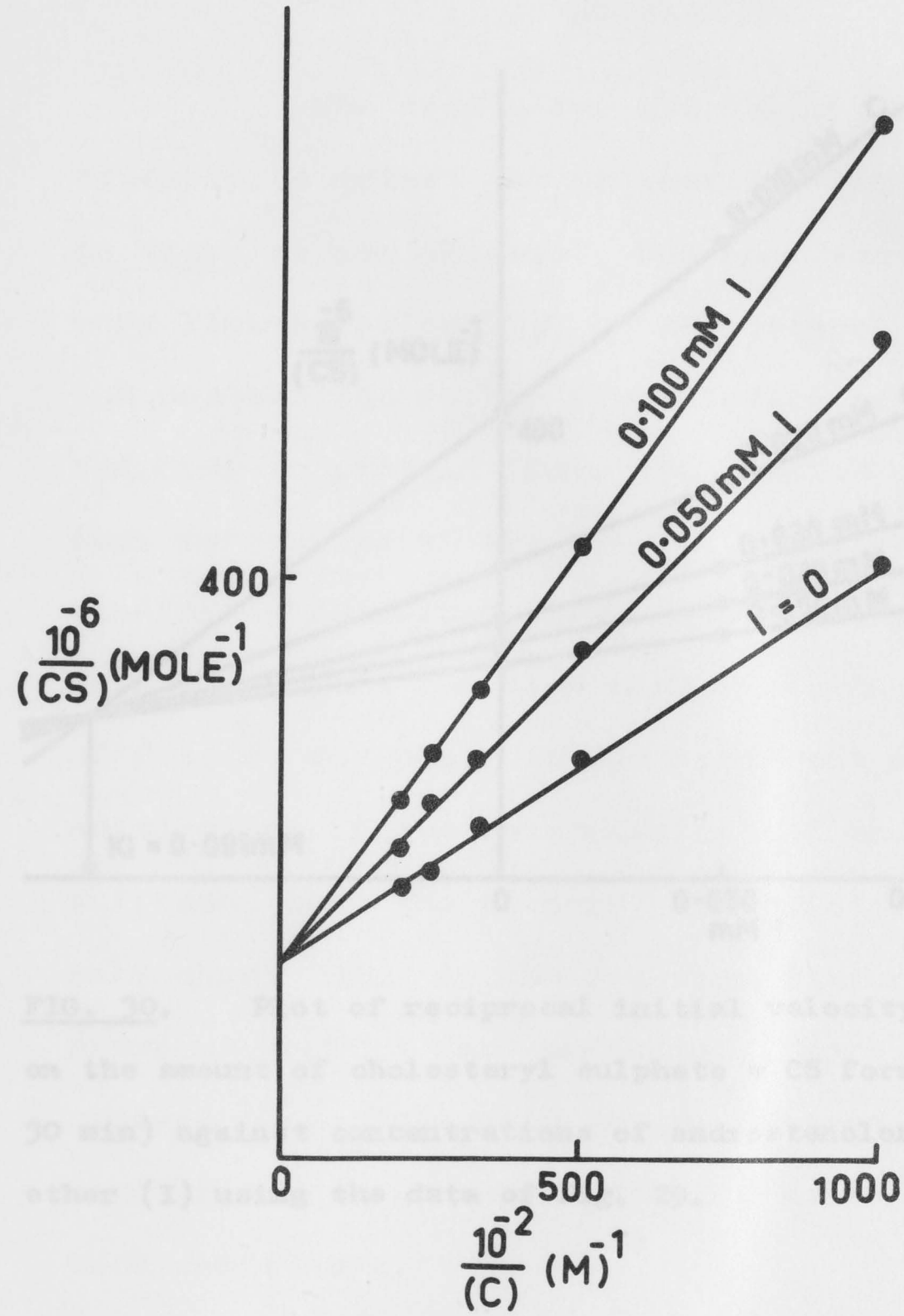


FIG. 29. Plot of reciprocal initial velocity (based on the amount of cholesteryl sulphate = CS formed per 30 min) against reciprocal concentrations of cholesterol (C) at constant PAPS (0.100 mM), showing competitive inhibition by added androstenolone methyl ether (I).

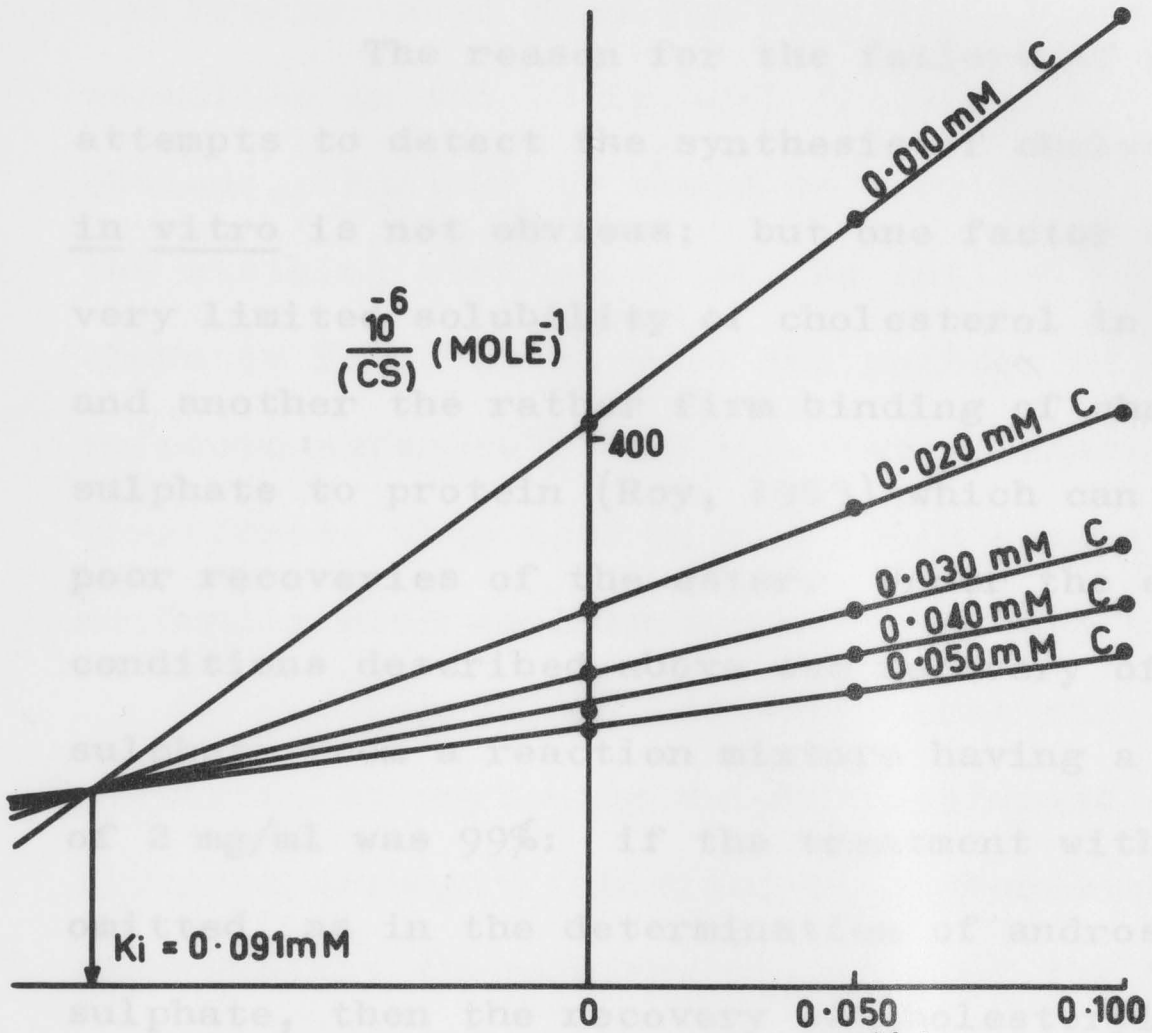


FIG. 30. Plot of reciprocal initial velocity (based on the amount of cholesteryl sulphate = CS formed per 30 min) against concentrations of androstenolone methyl ether (I) using the data of Fig. 29.

Discussion

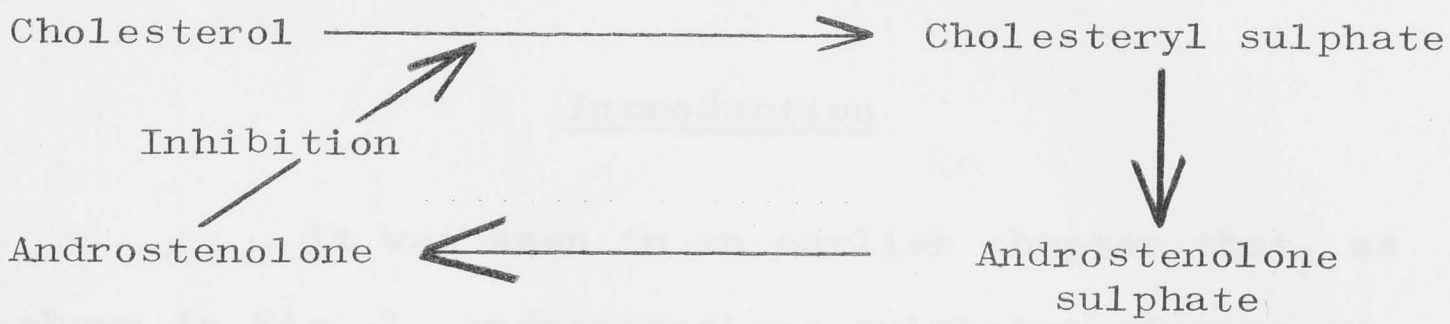
The reason for the failure of previous attempts to detect the synthesis of cholesteryl sulphate *in vitro* is not obvious; but one factor could be the very limited solubility of cholesterol in aqueous media and another the rather firm binding of cholesteryl sulphate to protein (Roy, 1963) which can cause very poor recoveries of the ester. Under the experimental conditions described above the recovery of cholesteryl sulphate from a reaction mixture having a protein content of 2 mg/ml was 99%: if the treatment with ethanol were omitted, as in the determination of androstenolone sulphate, then the recovery of cholesteryl sulphate was only about 40%. As the reaction mixtures used by previous workers were undoubtedly more concentrated in protein than those used in the present work, the recovery of cholesteryl sulphate would be very low under such conditions.

The fact that the reciprocal plots in Figs. 27-28 are rectilinear showed that there were no anamalous effects of changes in substrate concentration and, in particular, demonstrated that all the cholesterol remained in solution in the aqueous medium under the experimental conditions, that is, up to a cholesterol

concentration of at least 0.06 mM. If the cholesterol had precipitated from the reaction mixture then anomalies in the reciprocal plots would have become obvious. Further it would be tempting to suggest, from the striking similarity of the initial velocity patterns shown in Figs. 27-28 with the pattern of the phenol sulphotransferase reaction, that androstenolone sulphotransferase also acts with a rapid equilibrium random mechanism with noninteracting substrate binding sites.

Roy (1961) had shown that the aryl sulphamate synthesis by arylamine sulphotransferase of guinea pig liver is strongly inhibited by androstenolone methyl ether mainly through the reaction of the D ring of the 17-oxosteroid with the enzyme. The present work has shown that arylamine sulphotransferase probably has no separate existence in the guinea pig liver and its apparent activity is due to androstenolone and oestrone sulphotransferases. It is therefore possible that the inhibition noted here of the synthesis of cholesteryl sulphate by androstenolone sulphotransferase is due to the reaction of the D ring of the 17-oxosteroid with the enzyme. The inhibition then appears important because if cholesteryl sulphate is an obligatory intermediate in the conversion of cholesterol to androstenolone (Roberts et al., 1964a,b) then the latter

will control its own production by a feed back inhibition of androstenolone sulphotransferase as shown below.:-



Admittedly, the fact that the inhibition is competitive limits its potentiality as a control mechanism. Nevertheless, if the metabolism of cholesterol through its sulphate ester is of any physiological significance then this type of control is worthy of further investigation, specially because separate pools of cholesterol possibly occur (Savard, Marsh & Rice, 1965).

CHAPTER 9

EVIDENCE THAT ANDROSTENOLONE AND OESTRONE SULPHOTRANSFERASES INTERACT

Introduction

It was seen in an earlier chapter that, as shown in Fig. 1, androstenolone sulphotransferase is eluted from DEAE-Sephadex in a highly unsymmetrical way —— trailing backward, even to the extent of giving a second peak, in the androstenolone sulphotransferase fraction. For the sake of convenience Fig. 1 has been redrawn to give Fig. 31 in which the elution profile of androstenolone sulphotransferase alone has been shown at the highest (curve 1) and the lowest (curve 2) proportions of oestrone sulphotransferase. In curve 1 there is a prominent second peak of androstenolone sulphotransferase which disappears in curve 2 although the latter is seen to trail backward conspicuously. Although oestrone sulphotransferase is eluted from DEAE-Sephadex with an almost symmetrical peak, there is still some indication that this too trails in the forward direction in the main androstenolone sulpho-transferase fraction.

Adams (1967) has studied an oestrone

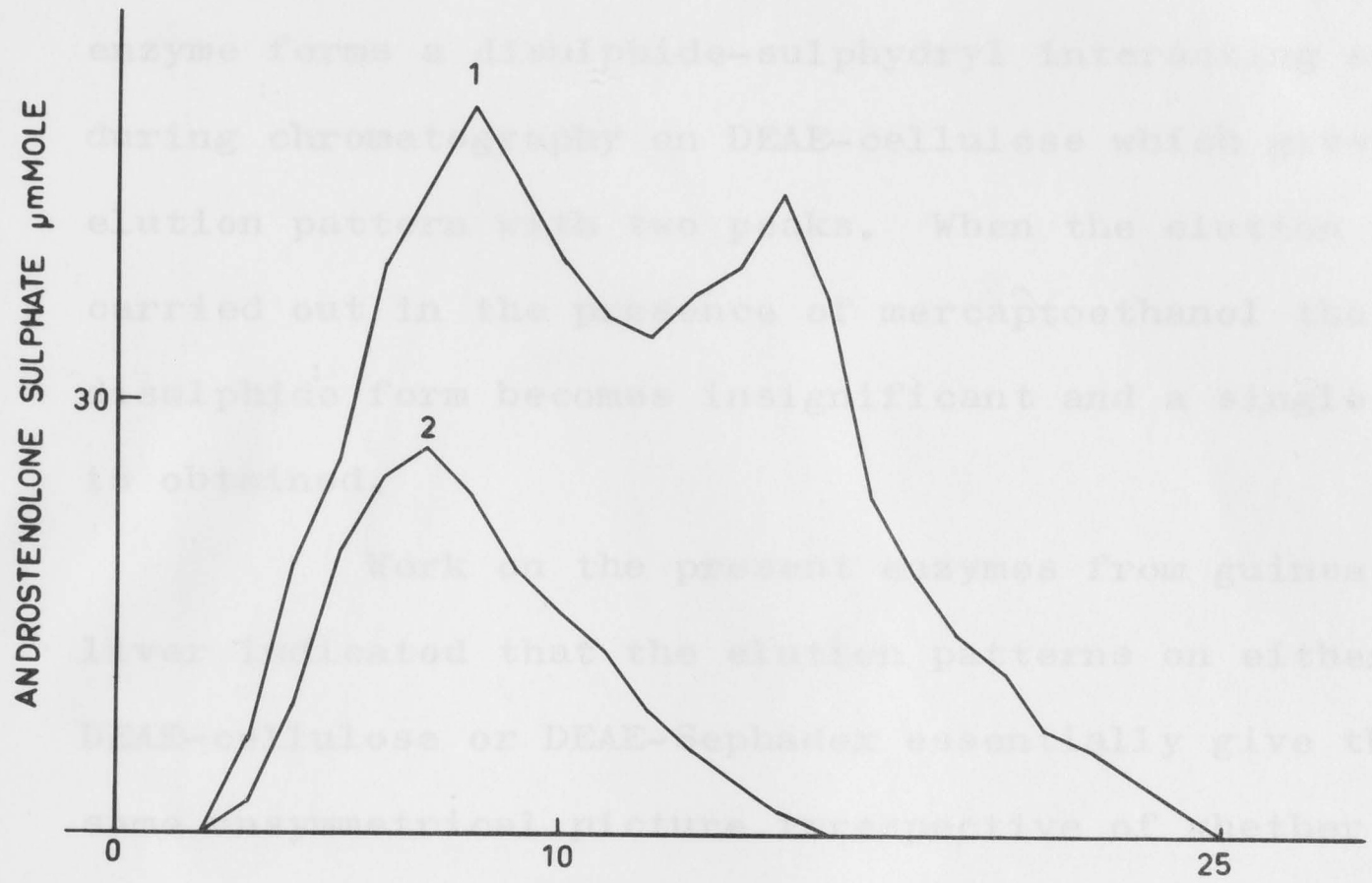


FIG. 31. The figure is redrawn from Fig. 1 to show the elution profile of androstenolone sulphotransferase alone at the highest (1) and the lowest (2) proportions of oestrone sulphotransferase.

sulphotransferase, free from androstenolone sulphotransferase, from ox adrenal gland and has found that this enzyme forms a disulphide-sulphydryl interacting system during chromatography on DEAE-cellulose which gives an elution pattern with two peaks. When the elution is carried out in the presence of mercaptoethanol the disulphide form becomes insignificant and a single peak is obtained.

Work on the present enzymes from guinea pig liver indicated that the elution patterns on either DEAE-cellulose or DEAE-Sephadex essentially give the same unsymmetrical picture irrespective of whether the elution is carried out in the presence or absence of mercaptoethanol. Fig. 32 shows elution profiles for androstenolone sulphotransferase on DEAE-Sephadex with (curve 2) and without (curve 1) mercaptoethanol. Both the curves are seen to trail backward.

Several questions which immediately arose were whether androstenolone and oestrone sulphotransferases reacted with each other and whether the reaction was through disulphide bond formation and, if disulphide bonds were involved, could the process be reversed by the addition of SO_3^{2-} ions, as Creeth & Nichol (1960) achieved with urease.

The interaction was not detectable on

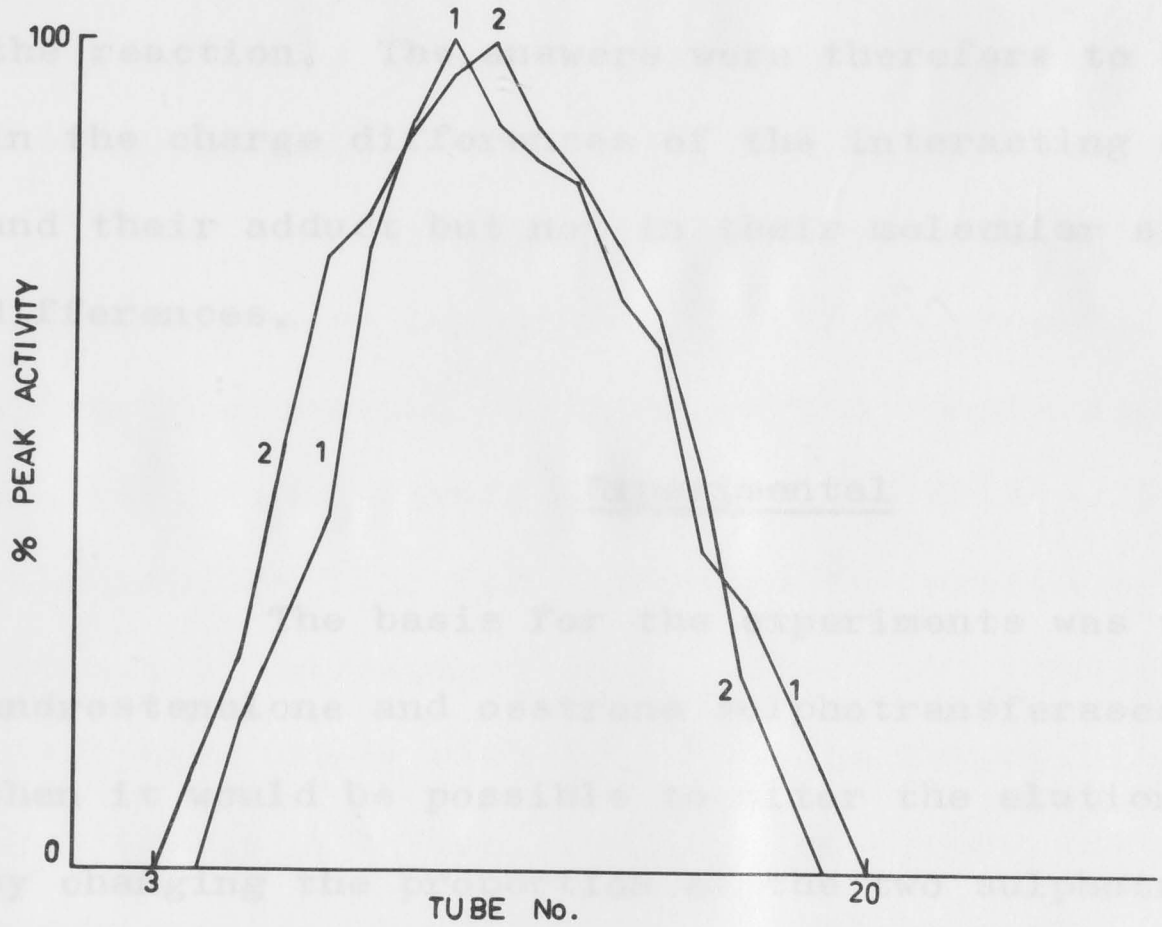


FIG. 32. Elution pattern of androstenolone sulphotransferase on DEAE-Sephadex in the presence (2) and absence (1) of mercaptoethanol. Activities are expressed relative to the activity in the peak fraction.

Sephadex G-200 since the peaks coincided symmetrically indicating that no change of molecular size accompanied the reaction. The answers were therefore to be sought in the charge differences of the interacting entitities and their adduct but not in their molecular size differences.

Experimental

The basis for the experiments was that if androstenolone and oestrone sulphotransferases reacted, then it would be possible to alter the elution patterns by changing the proportion of the two sulphotransferases in the preparations chromatographed.

The preparations for chromatography were made simply by mixing known amounts of the partially separated oestrone and androstenolone sulphotransferases and then dialysing the mixtures for seven days against the starting buffer. The exact composition of the mixtures was determined by assay. These prepared mixtures were chromatographed on DEAE-Sephadex.

For elution in the absence of SO_3^{2-} ions, the conditions were: elution with a linear concentration gradient formed from 100 ml of 0.01M EDTA-NaOH, pH 7.5, and 100 ml of 0.01M EDTA - 0.05M Tris - 0.30M sodium acetate, pH 7.5, both buffers containing 0.01M

mercaptoethanol, from a column (1.6 x 30 cm) of DEAE-Sephadex A-50. The enzymes were thus eluted with a linear ionic strength gradient (ionic strength rising from 0.06 to 0.40 in 200 ml.)

For studying the effect of SO_3^{2-} ions, the enzyme mixtures containing different proportions of androstenolone and oestrone sulphotransferases were dialysed exhaustively against 0.01M EDTA-0.05M sodium sulphite, pH 7.5, for 7 days in the presence of air and absence of mercaptoethanol. Since the reaction of disulphide bonds with SO_3^{2-} ions is reversible above pH 7 (Cecil & McPhee, 1955) it was found desirable to carry out the chromatography in the presence of SO_3^{2-} ions. The columns (1.6 x 30 cm) were run using a linear concentration gradient formed from 100 ml of the above buffer and 100 ml of 0.01M EDTA-0.05M sodium sulphite - 0.20M sodium acetate, pH 7.5. The enzymes were thus eluted with a linear ionic strength gradient (ionic strength rising from 0.21 to 0.41 in 200 ml.)

Results

The enzyme was not inhibited by sodium sulphite up to a SO_3^{2-} ion concentration of at least 0.05M and was stable after dialysis against 0.01M EDTA - 0.05M sodium sulphite, pH 7.5, for seven days.

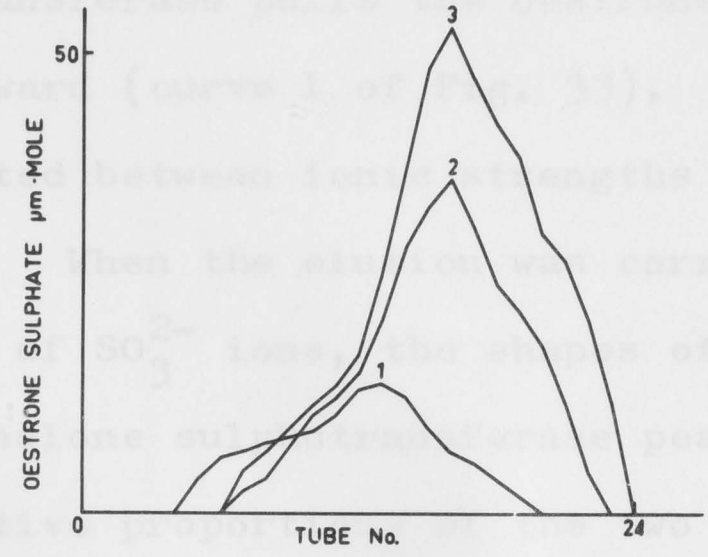
The activity of oestrone sulphotransferase before and after dialysis against SO_3^{2-} ions is tabulated below:-

Enzyme	Activity, millimicromole product/$\frac{1}{2}$ hr.	
	Before dialysis	After dialysis
Oestrone sulphotransferase	25.6	27.1

Fig. 33 shows the elution patterns of enzyme mixtures containing varying proportions of androstenolone and oestrone sulphotransferases in the absence of SO_3^{2-} ions. The oestrone sulphotransferase activity is shown in the top section and the corresponding androstenolone sulphotransferase activity in the bottom section of the figure. The ratios,

$$\frac{\text{androstenolone sulphotransferase activity}}{\text{oestrone sulphotransferase activity}},$$

for curves 1, 2 and 3 are 3.1, 1.6 and 1.2 respectively. Clearly the elution patterns of the two enzymes are dependent on these ratios. In the presence of a large amount of oestrone sulphotransferase, the androstenolone sulphotransferase peak is pushed backward giving an almost dual peak effect (curves 2 and 3 of Fig. 33),



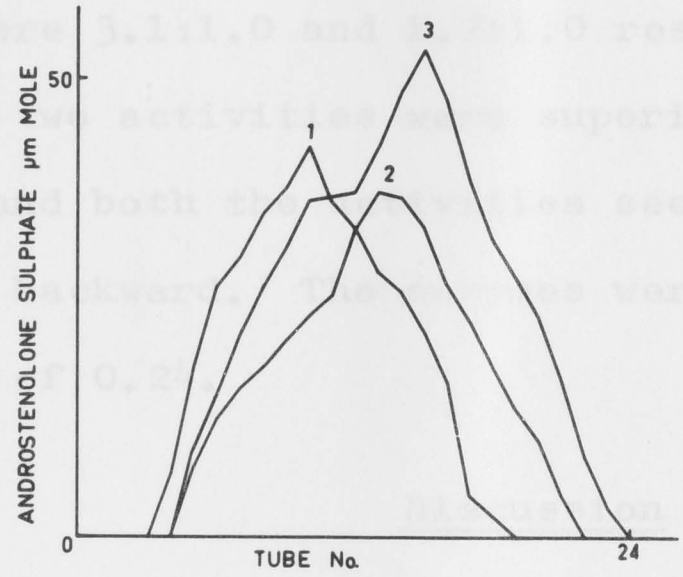


FIG. 33. Elution patterns of androstenolone and oestrone sulphotransferase mixtures on DEAE-Sephadex. The ratios $\frac{\text{androstenolone sulphotransferase activity}}{\text{oestrone sulphotransferase activity}}$ are 3.1, 1.6 and 1.2 for curves 1, 2 and 3 respectively. Activities are expressed as millimicromoles of products formed per 30 min.

whereas the presence of a large excess of androstenolone sulphotransferase pulls the oestrone sulphotransferase peak forward (curve 1 of Fig. 33). The enzyme peaks were eluted between ionic strengths of 0.14 and 0.16.

When the elution was carried out in the presence of SO_3^{2-} ions, the shapes of the oestrone and androstenolone sulphotransferase peaks did not vary with the relative proportions of the two enzymes as shown by curves 1 and 3 of Fig. 34 in which the proportion of androstenolone sulphotransferase to oestrone sulphotransferase were 3.1:1.0 and 1.2:1.0 respectively. In this case the two activities were superimposable during elution and both the activities seemed to trail slightly backward. The enzymes were eluted at an ionic strength of 0.24.

Discussion

Fig. 33 clearly shows that oestrone and androstenolone sulphotransferases interact. The interaction is not shown on Sephadex G-200 columns (operated under comparable conditions of pH and ionic strength) from which the enzymes are eluted with symmetrical peaks. This means that the different forms must either have the same molecular weights or be in slow reaction so that they do not separate

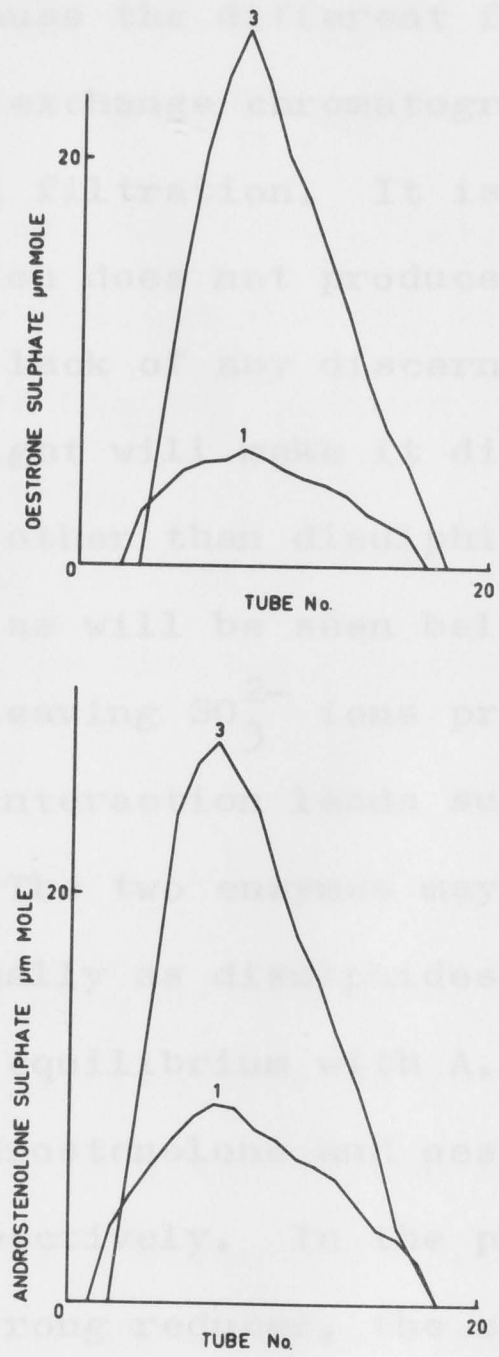


FIG. 34. Elution patterns of mixtures of sulphite treated androstenolone and oestrone sulphotransferases at former:latter ratios of 3.1:1.0 (curve 1) and 1.2:1.0 (curve 3) on DEAE Sephadex. Activities are expressed as in Fig. 33.

during gel filtration. The latter possibility is unlikely because the different forms do separate during anion exchange chromatography which takes no more time than gel filtration. It is probable therefore that the interaction does not produce any change in molecular weight. The lack of any discernible effect on the molecular weight will make it difficult to visualize any reaction other than disulphide/sulphydryl interchange, and, as will be seen below, the fact that disulphide-cleaving SO_3^{2-} ions presumably help to abolish the interaction lends support to this contention. The two enzymes may therefore be considered to exist normally as disulphides A.S - S.A and E.S - S.E in equilibrium with A.SH and E.SH where A and E are the androstenolone and oestrone sulphotransferase residues respectively. In the presence of mercaptoethanol, a strong reducer, the equilibrium is shifted towards the formation of A.SH and E.SH. The reactions are shown below:

(1) A.S - S.A + [2H] $\rightleftharpoons$ 2A.SH

(11) E.S - S.E + [2H] $\rightleftharpoons$ 2E.SH

The reduced forms can react with each other and with the disulphides to form mixed disulphides as follows.:-

(III) A.SH + E.SH $\rightleftharpoons$ A.S - S.E + [2H]

(IV) A.S-S.A + E.SH $\rightleftharpoons$ A.S - S.E + A.SH

(V) E.S-S.E + A.SH $\rightleftharpoons$ A.S - S.E + E.SH

Reactions (III), (IV) and (V) explain the change of the elution pattern with the relative proportions of the two enzymes present in the mixture for fractionation.

The role of SO_3^{2-} ions is to cleave the disulphide bonds and the reaction at pH 7.5 can be represented by

(VI) R.S - S.R' + SO_3^{2-} + $2H^+$ $\rightleftharpoons$ R.S.SO_3^- + R'SH + H^+

where R and R' can be either of the two residues A or E. Although the reaction is reversible above pH 7, all the disulphide form can be converted completely into S-sulphonate by prolonged dialysis against SO_3^{2-} ions in the presence of air or an oxidizing agent, because oxidation converts the sulphydryl form to disulphide which in turn reacts with more SO_3^{2-} ions. The cycle continues till only the S-sulphonate form remains. Fig. 34 shows that the elution patterns of the sulphite treated enzymes were probably independent of the relative amounts of the two enzymes present in the mixture fractionated although the two activities were

superimposed. These results may be interpreted as showing that the two enzymes are converted to the S-sulphonate forms, A.S.SO_3^- and E.S.SO_3^-, having similar pK values, so that they are eluted at the same ionic strength on Sephadex anion exchanger. The S-sulphonate forms of the enzymes contain an additional ionizing group, - S.SO_3^-, which cannot occur in the untreated form. Hence the sulphite modified enzymes will be more strongly adsorbed on an anion exchanger than the unmodified forms. The results that sulphite treated androstenolone and oestrone sulphotransferases are eluted from DEAE-Sephadex at an ionic strength of 0.24 (this value could be in error, because the enzymes were eluted too near the void volume of the column) against 0.15 for the untreated enzymes are consistent with the formation of enzyme - S-sulphonate by treatment with SO_3^{2-} ions. These results also suggest that if the two enzymes are sulphydryl-containing proteins, then the SH groups are not in the active centres.

It appears therefore that the interaction shown by oestrone and androstenolone sulphotransferases involves the participation of disulphide/sulphydryl interchange. For lack of time, experiments could not be performed to confirm this hypothesis. The obvious experiment which needs to be done is to convert the

enzymes quantitatively to S-sulphonates which must be stable in the absence of mercaptoethanol. Chromatography of the modified enzymes on DEAE-Sephadex under standard preparative conditions will then enable a direct comparison to be made of the ionic nature of the normal and modified enzymes.

GENERAL DISCUSSION

It was seen in the foregoing chapters that phenol, androstenolone and oestrone sulphotransferases quantitatively form the major part of the sulphotransferases, so far studied, of guinea pig liver; and that a phenol sulphotransferase can be obtained free from steroid sulphotransferases, whereas androstenolone and oestrone sulphotransferases can only be partially separated and remain associated with other types of sulphotransferase activity. The purifications achieved for these enzymes in the present work are tabulated below and for comparison the corresponding figures are included for choline sulphotransferase (Orsi & Spencer, 1964) which is the only other sulphotransferase for which the necessary data have been published. As with choline sulphotransferase, the main difficulty in the further purification of the present enzymes was their great instability.

Present Work

Enzyme source	Guinea pig liver		
Sulphotransferase type	Andros-tenolone	Oestrone	Phenol
Purification from crude extract	20 fold	70 fold	210 fold
Specific activity of purified preparation, μm mole product/10 min per mg protein.	28	35	350

Orsi & Spencer

Enzyme source	Aspergillus nidulans
Sulphotransferase type	Choline
Purification from crude extract	2.8 fold
Specific activity of purified preparation, μm mole product/ 10 min per mg protein.	0.008

The lack of accurate data on the sulphotransferases is most obvious and surprising. Despite the great interest of the enzymes in steroid biochemistry few attempts have been made to purify them and the information which is available on the distribution and activities in different tissues has been obtained under arbitrary conditions, often using unknown concentrations of PAPS as the sulphate donor. This applies not only to the important exploratory work

of Boström and his group (Boström, Franksson & Wengle, 1964; Wengle, 1966), but also to that of Nose & Lipmann (1958), Adams (1963a), Holcenberg & Rosen (1965) etc. From rat liver, Carroll & Spencer (1964) claimed to have separated two groups of sulphotransferases, one of which catalyzed the formation of androstenolone sulphate and p-nitrophenyl sulphate and the other of alkyl sulphates and aryl sulphamates. This work was reported in only a preliminary communication in which a 1,000 fold purification was claimed, but no further details have since appeared. Apparently difficulties have been found in repeating the work (Roy, personal communication). Nose & Lipmann (1958) partially separated the sulphotransferases of rat liver that were responsible for the formation of androstenolone sulphate, oestrone sulphate and p-nitrophenyl sulphate; but they did not further characterize the enzymes. Indeed the properties shown in two papers are contradictory: Lipmann (1958) showed the sulphotransferases moving towards the cathode on electrophoresis on Geon 426, while Nose & Lipmann (1958) reported their migration towards the anode under apparently identical conditions: in both the cases androstenolone sulphotransferase moved faster than phenol sulphotransferase. Only the former data presented by Lipmann

(1958) are consistent with the behaviour on DEAE-Sephadex of the enzymes used in the present studies.

It seems that the sulphotransferases of liver may not be as numerous as has been believed in the past. In guinea pig liver there certainly exist a phenol sulphotransferase, an androstenolone sulphotransferase, an oestrone sulphotransferase and a desoxycorticosterone sulphotransferase, the last named possibly also acting as a testosterone sulphotransferase. It is also probable that androstenolone and oestrone sulphotransferases can both accept p-nitrophenol and 2-naphthylamine as substrates and if these enzymes do show this multiple specificity then the existence of a separate arylamine sulphotransferase is doubtful. The strong inhibition of arylamine sulphotransferase activity by androstenolone methyl ether first noted by Roy (1960a) has been confirmed and this finding, coupled with the idea that androstenolone sulphotransferase can act as arylamine sulphotransferase, adds more weight to Roy's (1964) observation that the above inhibition is allosteric. Recently Adams (1967) has studied an oestrone sulphotransferase from ox adrenal gland. This enzyme has been found to be free from phenol sulphotransferase and arylamine sulphotransferase activity, so that it must be different from the oestrone sulphotransferase of

guinea pig liver, unless it is assumed that, similar to the interactions between androstenolone and oestrone sulphotransferases, a true arylamine sulphotransferase and phenol sulphotransferase can associate with both the above steroid sulphotransferases. However the findings in Chapters 3 and 4 that the ratios of the amount of 2-naphthyl sulphamate synthesized by variable mixtures of androstenolone and oestrone sulphotransferases during their elution from DEAE-Sephadex, to the total amount of androstenolone and oestrone sulphates formed are constant and that 17-oxosteroids powerfully inhibit 2-naphthyl sulphamate synthesis support the initial argument that androstenolone and oestrone sulphotransferases can themselves act as arylamine and phenol sulphotransferases.

The present work also demonstrates for the first time that cholesterol can be sulphurylated *in vitro* by one of the sulphotransferases, most probably by androstenolone sulphotransferase. Although the possibility of the existence of a specific cholesterol sulphotransferase cannot be excluded, there is so far no evidence to suggest that a separate cholesterol sulphotransferase exists. The failure of previous workers (Schneider & Lewbart, 1956; Nose & Lipmann, 1958) to detect this synthesis *in vitro* must

have been caused by the high protein content in the reaction mixtures (about 10 mg/ml) used by them, for cholesteryl sulphate is strongly bound to protein (Roy, 1963). In fact the recovery of cholesteryl sulphate from a reaction mixture containing 2 mg/ml of protein was found to be only 40% when steps were not taken to remove proteins by alcohol precipitation. This demonstration of the formation of cholesteryl sulphate in vitro seems to be important, since as pointed out by Baulieu et al. (1965), the previous failure to detect the synthesis made it difficult to visualize cholesteryl sulphate as a possible starting point for the biosynthesis of androstenolone sulphate and so of oestrogens.

The specificity of the steroid sulphotransferases is obscure and any definite conclusions must be dependent on the complete separation of the component enzymes, a procedure which will probably be complicated by mutual interactions as in the case of androstenolone and oestrone sulphotransferases. A useful procedure might be to take as starting material a tissue rich in one particular sulphotransferase: under such conditions any interactions would be minimized and purification might be simpler. Liver may therefore not be the tissue of choice for further work as is perhaps shown by

Adams' (1967) purification of oestrone sulphotransferase from ox adrenal, a tissue apparently containing only relatively small amounts of the other sulphotransferases. This relative purity might, of course, be outweighed by the fact that only much smaller amounts of tissue would be available for processing.

Undoubtedly the main problem about the steroid sulphotransferases is that of their exact physiological role in steroid metabolism - there can be no doubt that they do have such a role. The sulphurylation of cholesterol has been shown by Roberts et al. (1964a,b) to be a preliminary to further metabolism, and the sulphurylation of androstenolone has been cited as a means of maintaining a long acting transport form of steroid which can be metabolized further in the target tissues (Baulieu et al., 1965). The possibility of control of steroid metabolism at this stage must not be overlooked (Banerjee & Roy, 1967).

It was discussed earlier that the synthesis of p-nitrophenyl sulphate is catalyzed by at least three enzymes, the main one being a specific phenol sulphotransferase and the other two being steroid sulphotransferases. The phenol sulphotransferase has a pH optimum between 5.6 and 5.8 and does not require

added Mg^{2+} ions for activity, whereas the other two enzymes have flat pH optima between 6.5 and 7.5 and require added Mg^{2+} ions for maximal activity. If this complex situation is typical of aryl sulphate synthesis by enzymes from mammalian liver, then many of the inconsistencies in previous work become explicable. Such inconsistencies are, for instance, the requirement for Mg^{2+} ions claimed by Segal (1955) but disputed by Gregory & Lipmann (1957) or the positions of the pH optimum which have variously been reported between 6 and 8.5. These conflicting data are immediately explained if participation of more than one enzyme is assumed. Since the data of Yount, Simchuk, Yu & Kottke (1966) show that PAPS is only weakly bound to Mg^{2+} ions, the true substrate for the sulphotransferases is likely to be free PAPS and not a Mg-PAPS complex. The reasons why the steroid sulphotransferases require Mg^{2+} ions are not obvious, unless it is assumed that the enzymes react with Mg^{2+} ions directly. If this is so, then it is likely that the steroid sulphotransferases bind Mg^{2+} ions weakly so that they are removed by dialysis against EDTA, whereas the phenol sulphotransferase binds Mg^{2+} ions strongly and these are not removed by dialysis against EDTA. Perhaps Mg^{2+} ions are required

to stabilize one particular conformation of the protein molecules.

The kinetic studies on phenol sulphotransferase using p-nitrophenol (NP) as acceptor have shown that the enzyme catalyzes a rapid equilibrium random reaction in which an inactive ternary complex, E.NP.PAP, can form (Banerjee & Roy, 1966b, and Chapter 7). Further the combining sites for the two substrates (NP and PAPS) are independent, so that the presence of one substrate does not affect the combination of the other with the enzyme. The rapid equilibrium random mechanism with independent substrate binding sites seems to be quite general of the sulphotransferases, because androstenolone sulphotransferase probably also has a similar reaction mechanism (Chapter 8). Further the results of Orsi & Spencer (1964) also indicate that choline sulphotransferase from Aspergillus nidulans has a similar reaction mechanism with independent sites for choline and PAPS. The reaction mechanisms for the sulphotransferases are therefore quite similar to those of the phosphotransferases which are being extensively studied by many workers. The reaction catalyzed by ATP:creatine phosphotransferase has been shown by Morrison & James (1965) and by Morrison & Cleland (1966) to be a rapid equilibrium random type. Further the

latter authors used the isotope exchange technique (Boyer, 1959; Boyer & Silverstein, 1963) to show that during reaction a dead end ternary complex, enzyme - creatine - MgADP is formed. Similarly in the case of ATP:D-glucose 6-phosphotransferase, Fromm & Zewe (1962) and Fromm, Silverstein & Boyer (1964) have shown that the enzyme exhibits a rapid equilibrium random mechanism with the formation of a dead end ternary complex, enzyme - glucose - MgADP.

It was seen in Chapter 7 that phenol sulphotransferase did not significantly participate in the transsulphurylation reaction in the presence of PAP, p-nitrophenyl sulphate and 2-naphthol. This would mean that the enzyme has very different kinetic properties from those of the phenol sulphotransferase studied by Gregory & Lipmann (1957) and by Brunngraber (1958). In addition, the finding of Gregory & Lipmann (1957) that p-nitrophenyl sulphate could transfer the sulphuryl group only to phenols and not to other potential acceptors, such as steroids, seemed to indicate that the PAPS remained bound and did not dissociate from the enzyme. The finding of Wortman (1961) that a mucopolysaccharide sulphotransferase from beef cornea could utilize p-nitrophenyl sulphate as sulphate donor in the presence of PAP and phenol sulphotransferase

suggested, on the other hand, either that PAPS must dissociate from this phenol sulphotransferase or else that the enzyme exhibits dual specificity. The former is more likely because in the case of phenol sulphotransferase studied here, it is found that PAPS does dissociate from the enzyme with a dissociation constant for the enzyme - PAPS complex of 0.04 mM.

From the rather limited specificity studies so far made with phenol sulphotransferase it seems that a phenol can only be a substrate if it is a simple benzene derivative or, in the case of polycyclic phenols, if the ring adjacent to the phenolic ring is either aromatic or quinonoid. All the phenols listed in Table 12, and in addition m-aminophenol, are sulphurylated by phenol sulphotransferase. Further it was found by Jones (1966) using the enzyme samples sent from here that phenol sulphotransferase catalyzed the sulphurylation of tyrosine methyl ester and the K_m for the acceptor was 5 mM at pH 7.5. The pH optimum for this transfer was about 8.6 whereas that for the transfer to p-nitrophenol was 6.0. The difference in the two pH optima are explained by the fact that tyrosine derivatives are sulphurylated only when the amino group is unprotonated, that is at alkaline pH (Segal & Mologne, 1959). Jones (1966) also found that

both the androstenolone and oestrone sulphotransferases sulphurylated tyrosine methyl ester possibly through the participation of the associated phenol sulphotransferase activity. This relatively low specificity might have been predicted from the very wide range of phenols known to form sulphate esters *in vivo* (Williams, 1959). With the fairly broad specificity, it seems reasonable to assume that phenol sulphotransferase will catalyze the sulphurylation of thyroxine, serotonin and triiodothyronine : indeed, these could even be physiological substrates for the enzyme.

Vitamin A has been implicated by SubbaRao & Ganguly (1964) and Carroll & Spencer (1965b) in sulphotransferase activity. Unfortunately, in the present work no definite conclusions could be drawn on this effect of vitamin A : the addition of retinol (vitamin A) or retinyl acetate (vitamin A acetate) was without any effect on the activity. Nevertheless the possibility exists that the sulphotransferases studied here contained enough vitamin A, or a derivative thereof, to exhibit their full activity; but it should be noted that the purest preparation of phenol sulphotransferase available did not show any significant absorbance around 400 mμ where any highly unsaturated derivative of a carotenoid would be expected to absorb

strongly. On the other hand, the instability of the sulphotransferases towards acetone treatment suggests that a lipid factor may be involved in their structures. A definitive answer to this problem would therefore seem to hinge on the isolation and characterization of the suspected lipid cofactor from the pure enzymes.

It seems obvious that further work should be done to attempt to purify phenol sulphotransferase. The first step in the purification will obviously be to obtain a stable enzyme form. It may therefore be necessary to try if the enzyme can be stabilized by sulphite treatment. When a highly purified preparation is available an unambiguous answer to the role of vitamin A in sulphotransferase activity can be given.

As regards the steroid sulphotransferases, useful specificity studies can only be carried out if pure preparations are available. With such preparations, much useful information would be obtained from specificity studies, because steroids have rather rigid structures which can exist in many stable stereoisomeric forms. The study of the ability of such isomers to act as substrates would be expected to throw considerable light on the conformation of the enzyme around the active centre.

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+ These are abstracts of papersread at scientific meetings.